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GENETIC SEQUENCES ENCODING FLAVONOID PATHWAY ENZYMES AND USES THEREFOR

Field of the Invention

The present invention relates generally to genetic sequences encoding flavonoid pathway metabolising enzymes and more particularly to flavonoid 3'-hydroxylase (hereinafter referred to as "F3'H") or derivatives thereof and their use in the manipulation of pigmentation in plants and other organisms.

Bibliographic details of the publications referred to by the author in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs) for the nucleotide and amino acid sequences referred to in the specification and claims are defined following the bibliography. A summary of the SEQ ID NOs, and the sequences to which they relate, is provided prior to the Examples.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Description of the Related Art

The rapidly developing sophistication of recombinant DNA technology is greatly facilitating research and development in a range of biotechnology related industries. The horticultural industry has become a recent beneficiary of this technology which has contributed to developments in disease resistance in plants and flowers exhibiting delayed senescence after cutting. Some attention has also been directed to manipulating flower colour.

The flower industry strives to develop new and different varieties of flowering plants. An effective way to create such novel varieties is through the manipulation of flower colour. Classical breeding techniques have been used with some success to produce a wide range of colours for most of the commercial varieties of flowers. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for

a single species to have a full spectrum of coloured varieties. In addition, traditional breeding techniques lack precision. The aesthetic appeal of the flower is a combination of many factors such as form, scent and colour; modification of one character through hybridization can often be at the expense of an equally valuable feature. The ability to genetically engineer precise colour changes in cutflower and ornamental species would offer significant commercial opportunities in an industry which has rapid product turnover and where novelty is an important market characteristic.

Flower colour is predominantly due to two types of pigment: flavonoids and carotenoids. Flavonoids contribute to a range of colours from yellow to red to blue. Carotenoids impart an orange or yellow tinge and are commonly the major pigment in yellow or orange flowers. The flavonoid molecules which make the major contribution to flower colour are the anthocyanins which are glycosylated derivatives of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin, and are localised in the vacuole. The different anthocyanins can produce marked differences in colour. Flower colour is also influenced by co-pigmentation with colourless flavonoids, metal complexation, glycosylation, acylation and vacuolar pH (Forkmann, 1991).

The biosynthetic pathway for the flavonoid pigments (hereinafter referred to as the "flavonoid pathway") is well established and is shown in Figures 1a and 1b (Ebel and Hahlbrock, 1988; Hahlbrock and Grisebach, 1979; Wiering and De Vlaming, 1984; Schram *et al.*, 1984; Stafford, 1990; Van Tunen and Mol, 1990; Dooner *et al*, 1991; Martin and Gerats, 1993; Holton and Cornish, 1995). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2′,4,4′,6′, tetrahydroxy-chalcone, is normally rapidly isomerized to produce naringenin by the enzyme chalcone flavanone isomerase (CHI). Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The pattern of hydroxylation of the B-ring of DHK plays a key role in determining petal colour. The B-ring can be hydroxylated at either the 3′, or both the 3′ and 5′ positions, to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. Two key enzymes involved in this pathway are flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase, both of the cytochrome P450 class. Cytochrome P450 enzymes are widespread in nature and genes have been isolated and sequenced from vertebrates, insects, yeasts, fungi, bacteria and plants.

Flavonoid 3′-hydroxylase acts on DHK to produce DHQ and on naringenin to produce eriodictyol. Reduction and glycosylation of DHQ produces the cyanidin-glycoside and peonidin-glycoside pigments which, in many plant species (for example rose, carnation and chrysanthemum), contribute to red and pink flower colour. The synthesis of these anthocyanins can also result in other flower colours. For example, blue cornflowers contain cyanin. The ability to control flavonoid 3′-hydroxylase activity, or other enzymes involved in the flavonoid pathway, in flowering plants would provide a means to manipulate petal colour. Different coloured versions of a single cultivar could thereby be generated and in some instances a single species would be able to produce a broader spectrum of colours.

A nucleotide sequence (referred to herein as SEQ ID NO:26) encoding a petunia flavonoid 3′-hydroxylase has been cloned (see International Patent Application No. PCT/AU93/00127 [WO 93/20206]). However, this sequence was inefficient in its ability to modulate the production of 3′-hydroxylated anthocyanins in plants. There is a need, therefore, to identify further genetic sequences encoding flavonoid 3′-hydroxylases which efficiently modulate the hydroxylation of flavonoid compounds in plants. More particularly, there is a need to identify further genetic sequences encoding flavonoid 3′-hydroxylases which efficiently modulate the production of 3′-hydroxylated anthocyanins in plants.

Summary of the Invention

In accordance with the present invention, genetic sequences encoding flavonoid 3′-hydroxylase have been identified and cloned. The recombinant genetic sequences of the present invention permit the modulation of expression of genes encoding this enzyme by, for

example, *de novo* expression, over-expression, suppression, antisense inhibition and ribozyme activity. The ability to control flavonoid 3′-hydroxylase synthesis in plants permits modulation of the composition of individual anthocyanins as well as alteration of relative levels of flavonols and anthocyanins, thereby enabling the manipulation of tissue colour, such as petals, leaves, seeds and fruit. The present invention is hereinafter described in relation to the manipulation of flower colour but this is done with the understanding that it extends to manipulation of other plant tissues, such as leaves, seeds and fruit.

Detailed Description of the Invention

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding a flavonoid 3′-hydroxylase or a derivative thereof wherein said flavonoid 3′-hydroxylase or its derivative is capable of more efficient modulation of hydroxylation of flavonoid compounds in plants than is a flavonoid 3′-hydroxylase encoded by the nucleotide sequence set forth in SEQ ID NO:26.

Efficiency as used herein relates to the capability of the flavonoid 3′-hydroxylase enzyme to hydroxylate flavonoid compounds in a plant cell. This provides the plant with additional substrates for other enzymes of the flavonoid pathway able to further modify this molecule, via, for example, glycosylation, acylation and rhamnosylation, to produce various anthocyanins which contribute to the production of a range of colours. The modulation of 3′-hydroxylated anthocyanins is thereby permitted. Efficiency is conveniently assessed by one or more parameters selected from: extent of transcription, as determined by the amount of mRNA produced; extend of hydroxylation of naringenin and/or DHK; extent of translation of mRNA, as determined by the amount of translation product produced; extent of production of anthocyanin derivatives of DHQ or DHM; the extent of effect on tissue colour, such as flowers, seeds, leaves or fruits.

Another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides which maps to the genetic locus designated Ht1 or Ht2 in petunia, or to equivalent such loci in other flowering plant species, and wherein said isolated nucleic acid molecule encodes, or is complementary to a sequence which encodes,

a flavonoid 3'-hydroxylase.

A further aspect of the present invention contemplates an isolated nucleic acid molecule comprising a sequence of nucleotides which corresponds to the genetic locus designated Ht1 or Ht2 in petunia, or to loci in other flowering plant species which contain sequences which control production of 3'-hydroxylated flavonoids, and wherein said isolated nucleic acid molecule encodes a flavonoid 3'-hydroxylase or a derivative thereof which is capable of more efficient conversion of DHK to DHQ in plants than is the flavonoid 3'-hydroxylase set forth in SEQ ID NO:26.

In accordance with the above aspects of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:1 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:1 under low stringency conditions.

In a related embodiment, there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:3 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:3 under low stringency conditions.

In another related embodiment, the present invention is directed to a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:5 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:5 under low stringency conditions.

Yet another related embodiment provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:7 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:7 under low stringency conditions.

Still yet a further embodiment of the present invention relates to a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:9 or having at least about 60% similarity to the coding region thereof or capable of hybridising to the sequence set forth in SEQ ID NO:9 under low stringency conditions.

In another further embodiment, there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:14 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:14 under low stringency conditions.

In yet another further embodiment, the present invention is directed to a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:16 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:16 under low stringency conditions.

Still yet another further embodiment provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:18 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:18 under low stringency conditions.

Moreover, yet a further embodiment of the present invention relates to a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:20 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:20 under low stringency conditions.

Still yet another further embodiment is directed to a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:22 or having at least about 60% similarity thereto or capable of hybridising to the

sequence set forth in SEQ ID NO:22 under low stringency conditions.

In still yet another further embodiment, the present invention provides a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:24 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:24 under low stringency conditions.

In a particularly preferred embodiment there is provided an isolated nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:1 or having at least about 60% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:1 under low stringency conditions, wherein said nucleotide sequence maps to the genetic locus designated Ht1 or Ht2 in petunia, or to equivalent such loci in other flowering plant species, and wherein said isolated nucleic acid molecule encodes, or is complementary to a sequence which encodes, a flavonoid 3′-hydroxylase.

Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% to at least about 15% formamide and from at least about 1M to at least about 2M salt for hybridization, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% to at least about 30% formamide and from at least about 0.5M to at least about 0.9M salt for hybridization, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% to at least about 50% formamide and from at least about 0.01M to at least about 0.15M salt for hybridization, and at least about 0.01M to at least about 0.15M salt for washing conditions. Hybridization may be carried out at different temperatures and, where this occurs, other conditions may be adjusted accordingly.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid

sequence substantially as set forth in SEQ ID NO:2 or an amino acid sequence having at least about 50% similarity thereto.

In a related embodiment, there is provided a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:4 or an amino acid sequence having at least about 50% similarity thereto.

A further related embodiment of the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:6 or an amino acid sequence having at least about 50% similarity thereto.

Still another related embodiment provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:8 or an amino acid sequence having at least about 50% similarity thereto.

Yet still another related embodiment relates to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:10 or SEQ ID NO:11 or SEQ ID NO:12 or SEQ ID NO:13 or an amino acid sequence having at least about 50% similarity thereto.

In another further embodiment, there is provided a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:15 or an amino acid sequence having at least about 50% similarity thereto.

In yet another further embodiment, the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an

amino acid sequence substantially as set forth in SEQ ID NO:17 or an amino acid sequence having at least about 50% similarity thereto.

Still yet another further embodiment provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:19 or an amino acid sequence having at least about 50% similarity thereto.

Moreover, yet a further embodiment of the present invention relates to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:21 or an amino acid sequence having at least about 50% similarity thereto.

Still yet another further embodiment is directed to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:23 or an amino acid sequence having at least about 50% similarity thereto.

In still yet another further embodiment, the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:25 or an amino acid sequence having at least about 50% similarity thereto.

In a particularly preferred embodiment there is provided an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:2 or an amino acid sequence having at least about 50% similarity thereto, wherein said sequence of nucleotides maps to the genetic locus designated Ht1 or Ht2 in petunia, or to equivalent such loci in other flowering plant species, and wherein said isolated nucleic acid molecule encodes, or is complementary to a sequence which encodes, a flavonoid 3′-hydroxylase or a derivative

therof.

The term "similarity" as used herein includes exact identity between compared sequences, at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels.

The nucleic acid molecule defined by SEQ ID NO:1 encodes a flavonoid 3'-hydroxylase from petunia. Examples of other suitable F3'H genes are from carnation (SEQ ID NO:3), snapdragon (SEQ ID NO:5), arabidopsis (SEQ ID NO:7), arabidopsis genomic DNA clone (SEQ ID NO: 9), rose (SEQ ID NO:14), chrysanthemum (SEQ ID NO:16), torenia (SEQ ID NO:18), Japanese morning glory (SEQ ID NO:20), gentian (SEQ ID NO:22) and lisianthus (SEQ ID NO:24). Although the present invention is particularly exemplified by the aforementioned F3'H genes, the subject invention extends to F3'H genes from any species of plant provided that the F3'H gene has at least about 60% similarity at the nucleotide level to a nucleic acid molecule selected from SEQ ID NO:1 or 3 or 5 or 7 or 14 or 16 or 18 or 20 or 22 or 24, or at least about 50% similarity at the amino acid level to an amino acid molecule selected from SEQ ID NO: 2 or 4 or 6 or 8 or 10, 11, 12, 13 or 15 or 17 or 19 or 21 or 23 or 25. The subject invention further extends to F3'H genes from any species of plant provided that the F3'H gene has at least about 60% similarity at the nucleotide level to the coding region of SEQ ID NO:9.

The nucleic acid molecules of the present invention are generally genetic sequences in a non-naturally-occurring condition. Generally, this means isolated away from its natural state or synthesized or derived in a non-naturally-occurring environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro,* including genomic DNA fragments, recombinant or synthetic molecules and nucleic acids in combination with

heterologous nucleic acids. It also extends to the genomic DNA or cDNA or part thereof encoding F3'H or part thereof in reverse orientation relative to its or another promoter. It further extends to naturally-occurring sequences following at least a partial purification relative to other nucleic acid sequences.

The term "nucleic acid molecule" includes a nucleic acid isolate and a genetic sequence and is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or *via* a complementary series of bases, a sequence of amino acids in a F3'H. Such a sequence of amino acids may constitute a full-length F3'H or an active truncated form thereof or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme. The nucleic acid molecules contemplated herein also encompass oligonucleotides useful as genetic probes or as "antisense" molecules capable of regulating expression of the corresponding gene in a plant. An "antisense molecule" as used herein may also encompass a gene construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its own or another promoter. Accordingly, the nucleic acid molecules of the present invention may be suitable for use as cosuppression molecules, ribozyme molecules, sense molecules and antisense molecules to modulate levels of 3'-hydroxylated anthocyanins.

In one embodiment, the nucleic acid molecule encoding F3'H or various derivatives thereof is used to reduce the activity of an endogenous F3'H, or alternatively the nucleic acid molecule encoding this enzyme or various derivatives thereof is used in the antisense orientation to reduce activity of the F3'H. Although not wishing to limit the present invention to any one theory, it is possible that the introduction of the nucleic acid molecule into a cell results in this outcome either by decreasing transcription of the homologous endogenous gene or by increasing turnover of the corresponding mRNA. This may be achieved using gene constructs containing F3'H nucleic acid molecules or various derivatives thereof in either the sense or the antisense orientation. In a further alternative, ribozymes could be used to inactivate target nucleic acid molecules. Alternatively, the nucleic acid molecule encodes a functional F3'H and this is used to elevate levels of this enzyme in plants.

Reference herein to the altering of flavonoid F3'H activity relates to an elevation or reduction in activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. The level of activity can be readily assayed using a modified version of the method described by Stotz and Forkmann (1982) (see Example 7) or by assaying for the amount of F3'H product such as quercetin, cyanidin or peonidin as set forth in Example 5.

The present invention further extends to nucleic acid molecules in the form of oligonucleotide primers or probes capable of hybridizing to a portion of the nucleic acid molecules contemplated above, and in particular those selected from the nucleic acid molecules set forth in SEQ ID NOs: 1, 3, 5, 7, 9, 14, 16, 18, 20, 22 or 24 under high, preferably under medium and most preferably under low stringency conditions. Preferably the portion corresponds to the 5' or the 3' end of the F3'H gene. For convenience the 5' end is considered herein to define a region substantially between the 5' end of the primary transcript to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the 3' end of the primary transcript. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends.

The nucleic acid molecule or its complementary form may encode the full-length enzyme or a part or derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally-occurring enzyme and includes parts, fragments, portions, fusion molecules, homologues and analogues. In this regard, the nucleic acid includes the naturally-occurring nucleotide sequence encoding F3'H or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally-occurring sequence. A fusion molecule may be a fusion between nucleotide sequences encoding two or more F3'Hs, or a fusion between a nucleotide sequence encoding an F3'H and a nucleotide sequence encoding any other proteinaceous molecule. Fusion molecules are useful in altering substrate specificity.

A derivative of the nucleic acid molecule or its complementary form, or of a F3'H, of the present invention may also include a "part", whether active or inactive. An active or functional nucleic acid molecule is one which encodes an enzyme with F3'H activity. An active or functional molecule further encompasses a partially-active molecule; for example, an F3'H with reduced substrate specificity would be regarded as partially active. A derivative of a nucleic acid molecule may be useful as an oligonucleotide probe, as a primer for polymerase chain reactions or in various mutagenic techniques, for the generation of antisense molecules or in the construction of ribozymes. They may also be useful in developing co-suppression constructs. The nucleic acid molecule according to this aspect of the present invention may or may not encode a functional F3'H. A "part" may be derived from the 5' end or the 3' end or a region common to both the 5' and the 3' ends of the nucleic acid molecule.

Amino acid insertional derivatives of the F3'H of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 1 below.

TABLE 1

Suitable residues for amino acid substitutions

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Where the F3'H is derivatised by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.* (1989).

Other examples of recombinant or synthetic mutants and derivatives of the F3'H of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogues" and "derivatives" also extend to any chemical equivalents of the F3'H, whether functional or not, and also to any amino acid derivative described above. Where the "analogues" and "derivatives" of this aspect of the present invention are non-functional, they may act as agonists or antagonists of F3'H activity. For convenience, reference to "F3'H" herein includes reference to any derivatives, including parts, mutants, fragments, homologues or analogues thereof.

The present invention is exemplified using nucleic acid sequences derived from petunia, carnation, rose, snapdragon, arabidopsis, chrysanthemum, lisianthus, torenia, morning glory and gentian, since these represent the most convenient and preferred sources of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms. Examples of other plants include, but are not limited to, maize, tobacco, cornflower, pelargonium, apple, gerbera and african violet. All such nucleic acid sequences encoding directly or indirectly a flavonoid pathway enzyme and in particular F3'H, regardless of their source, are encompassed by the present invention.

The nucleic acid molecules contemplated herein may exist in either orientation alone or in combination with a vector molecule, for example an expression-vector. The term vector molecule is used in its broadest sense to include any intermediate vehicle for the nucleic acid molecule, capable of facilitating transfer of the nucleic acid into the plant cell and/or facilitating integration into the plant genome. An intermediate vehicle may, for example, be adapted for use in electroporation, microprojectile bombardment, *Agrobacterium*-mediated transfer or insertion via DNA or RNA viruses. The intermediate vehicle and/or the nucleic acid molecule contained therein may or may not need to be stably integrated into the plant genome. Such vector molecules may also replicate and/or express in prokaryotic cells. Preferably, the vector molecules or parts thereof are capable of integration into the plant genome. The nucleic acid molecule may additionally contain a promoter sequence capable of directing expression of the nucleic acid molecule in a plant cell. The nucleic acid molecule and promoter may also be introduced into the cell by any number of means such as those described above.

In accordance with the present invention, a nucleic acid molecule encoding a F3'H or a derivative or part thereof may be introduced into a plant in either orientation to allow, permit or otherwise facilitate manipulation of levels of production of mRNA in the cytoplasm and/or production of enzyme from the mRNA, thereby providing a means either to convert DHK and/or other suitable substrates, if synthesised in the plant cell, ultimately into anthocyanin derivatives of anthocyanidins such as cyanidin and/or peonidin, or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing F3'H activity. The production of mRNA in the cytoplasm and/or production of enzyme from the mRNA, is referred to herein as "expression". The production of anthocyanins contributes to the production of a red or blue flower colour. Expression of the nucleic acid molecule in either orientation in the plant may be constitutive, inducible or developmental, and may also be tissue-specific.

According to this aspect of the present invention there is provided a method for producing a transgenic plant capable of synthesizing F3'H or functional derivatives thereof, said method

comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding said F3'H, under conditions permitting the eventual expression of said nucleic acid molecule, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid molecule. The transgenic plant may thereby produce elevated levels of F3'H activity relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced endogenous or existing F3'H activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding F3'H, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid molecule.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced endogenous or existing F3'H activity, said method comprising altering the F3'H gene through modification of the endogenous sequences via homologous recombination from an appropriately altered F3'H gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

In accordance with these aspects of the present invention the preferred nucleic acid molecules are substantially as set forth in SEQ ID NO:1, 3, 5, 7, 14, 16, 18, 20, 22, 24, or the coding region of 9, or have at least about 60% similarity thereto, or are capable of hybridising thereto under low stringency conditions.

In a preferred embodiment, the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered flower colour, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid molecule into the F3'H

enzyme. Alternatively, said method may comprise stably transforming a cell of a suitable plant with a nucleic acid molecule of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the endogenous or existing F3'H. Preferably, the altered level would be less than the endogenous or existing level of F3'H activity in a comparable non-transgenic plant.

In a related embodiment, the present invention contemplates a method for producing a flowering plant exhibiting altered flower colour, said method comprising alteration of the F3'H gene through modification of the endogenous sequences *via* homologous recombination from an appropriately altered F3'H gene or derivative thereof introduced into the plant cell and regenerating the genetically modified plant from the cell.

The nucleic acid molecules of the present invention may or may not be developmentally regulated. Preferably, the modulation of levels of 3'-hydroxylated anthocyanins leads to altered flower colour which includes the production of red flowers or other colour shades depending on the physiological conditions of the recipient plant. By "recipient plant" is meant a plant capable of producing a substrate for the F3'H enzyme, or producing the F3'H enzyme itself, and possessing the appropriate physiological properties and genotype required for the development of the colour desired. This may include but is not limited to petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus, gerbera, apple, iris, lily, african violet, gentian, torenia and Japanese morning glory.

Accordingly, the present invention extends to a method for producing a transgenic plant capable of modulating levels of 3'-hydroxylated anthocyanins, said method comprising stably transforming a cell or group of cells of a suitable plant with a nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, F3'H or a derivative thereof, and regenerating a transgenic plant from said cell or cells.

One skilled in the art will immediately recognise the variations applicable to the methods of

the present invention, such as increasing or decreasing the level of enzyme activity of the enzyme naturally present in a target plant leading to differing shades of colours.

The present invention, therefore, extends to all transgenic plants containing all or part of the nucleic acid module of the present invention and/or any homologues or related forms thereof or antisense forms of any of these and in particular those transgenic plants which exhibit altered flower colour. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding F3'H. Generally, the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of the F3'H nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to seeds from such transgenic plants. Such seeds, especially if coloured, will be useful as proprietary tags for plants.

A further aspect of the present invention is directed to recombinant forms of F3'H. The recombinant forms of the enzymes will provide a source of material for research to develop, for example, more active enzymes and may be useful in developing *in vitro* systems for production of coloured compounds.

Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of use in modulating levels of 3'-hydroxylated anthocyanins in a plant or cells of a plant.

Yet a further aspect of the present invention provides flowers and in particular cut flowers, from the transgenic plants herein described, exhibiting altered flower colour.

Another aspect of the present invention is directed to a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding, a F3'H or a derivative thereof wherein said nucleic acid molecule is capable of being expressed in a plant cell. The term "expressed" is equivalent to the term "expression" as defined above.

The nucleic acid molecules according to this and other aspects of the invention allow, permit or otherwise facilitate increased efficiency in modulation of 3'-hydroxylated anthocyanins relative to the efficency of the pCGP619 cDNA insert contained in plasmid pCGP809, disclosed in International Patent Application No. PCT/AU93/00127 [WO 93/20206]. The term "plant cell" includes a single plant cell or a group of plant cells such as in a callus, plantlet or plant or parts thereof including flowers and seeds.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence of nucleotides encoding a F3'H, wherein the translation of the said nucleic acid molecule comprises the amino acid sequence RPPNSGA. Preferably, the translation of the said nucleic acid molecule comprises the amino acid sequence RPPNSGAXHXAYNYXDL and still more preferably the translation of the said nucleic acid molecule comprises the amino acid sequence RPPNSGAXHXAYNYXDL$[X]_n$GGEK, where X represents any amino acid and $[X]_n$ represents an amino acid sequence of from 0 to 500 amino acids.

The present invention is further described by reference to the following non-limiting Figures and Examples.

~~In the Figures:~~ Brief Description of the Drawings

1A-1B

Figures ~~1a and 1b~~ are schematic representations of the flavonoid biosynthesis pathways in *P. hybrida* flowers showing the enzymes and genetic loci involved in the conversions. Enzymes involved in the pathway have been indicated as follows: PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS= chalcone synthase; CHI= chalcone isomerase; F3H= flavanone 3-hydroxylase; F3'H= flavonoid 3'-hydroxylase; F3'5'H= flavonoid 3'5' hydroxylase; FLS= flavonol synthase; DFR= dihydroflavonol-4-reductase; ANS= anthocyanin synthase; 3GT= UDP-glucose: anthocyanin-3-glucoside; 3RT= UDP-rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase; ACT= anthocyanidin-3-rutinoside acyltransferase; 5GT= UDP-

glucose: anthocyanin 5- glucosyltransferase; 3' OMT= anthocyanin *O*-methyltransferase; 3', 5' OMT=anthocyanin 3', 5' *O*-methyltransferase. Three flavonoids in the pathway are indicated as: P-3-G= pelargonidin-3-glucoside; DHM=dihydomyricetin; DHQ=dihydroquercetin. The flavonol, myricetin, is only produced at low levels and the anthocyanin, pelargonidin, is rarely produced in *P. hybrida*.

Figure 2 is a diagrammatic representation of the plasmid pCGP161 containing a cDNA clone (F1) representing the cinnamate-4-hydroxylase from *P. hybrida*. ^{32}P-labelled fragments of the 0.7 kb EcoRI/XhoI fragment were used to probe the Old Glory Red petal cDNA library. For details, refer to Example 4. Abbreviations are as follows: Amp = the ampicillin resistance gene; ori = origin of replication; T3 = recognition sequence for T3 RNA polymerase; T7 = recognition sequence for T7 RNA polymerase. Restriction enzyme sites are also marked.

Figure 3 is a diagrammatic representation of the plasmid pCGP602 containing a cDNA clone (617) representing a flavonoid 3'5' hydroxylase (Hf1) from *P. hybrida*. ^{32}P-labelled fragments of the 1.6 kb BspHI/EspI fragment containing the Hf1 coding region were used to probe the Old Glory Red petal cDNA library. For details, refer to Example 4. Abbreviations are as follows: Amp = the ampicillin resistance gene; ori = origin of replication; T3 = recognition sequence for T3 RNA polymerase; T7 = recognition sequence for T7 RNA polymerase. Restriction enzyme sites are also marked.

Figure 4 is a diagrammatic representation of the plasmid pCGP175 containing a cDNA clone (H2) representing a flavonoid 3'5' hydroxylase (Hf2) from *P. hybrida*. ^{32}P-labelled fragments of the 1.3 kb EcoRI/XhoI and 0.5 kb XhoI fragments which together contain the Hf2 coding region were used to probe the Old Glory Red petal cDNA library. For details, refer to Example 4. Abbreviations are as follows: Amp = the ampicillin resistance gene; ori = origin of replication; T3 = recognition sequence for T3 RNA polymerase; T7 = recognition sequence for T7 RNA polymerase. Restriction enzyme sites are also marked.

Figure 5 is a diagrammatic representation of the plasmid pCGP619 containing the 651 cDNA clone representing a cytochrome P450 from *P. hybrida*. ^{32}P-labelled fragments of the 1.8 kb EcoRI/XhoI fragment were used to probe the Old Glory Red petal cDNA library. For details, refer to Example 4. Abbreviations are as follows: Amp = the ampicillin resistance gene; ori = origin of replication; T3 = recognition sequence for T3 RNA polymerase; T7 = recognition sequence for T7 RNA polymerase. Restriction enzyme sites are also marked.

Figure 6 is a representation of an autoradiograph of an RNA blot probed with ^{32}P-labelled fragments of the OGR-38 cDNA clone contained in pCGP1805 (see Example 6). Each lane contained a 20 μg sample of total RNA isolated from the flowers or leaves of plants of a V23 (htl/htl) x VR (Htl/htl) backcross population. A 1.8 kb transcript was detected in the VR-like (Htl/htl) flowers that contained high levels of quercetin (Q+)(lanes 9 - 14). The same size transcript was detected at much lower levels in the V23-like (htl/htl) flowers that contained little or no quercetin (Q-) (lanes 3-8). A reduced level of transcript was also detected in VR leaves (lane 1) and V23 petals (lane 2). This is described in Example 5.

Figure 7 is a diagrammatic representation of the yeast expression plasmid pCGP1646 (see Example 7). The OGR-38 cDNA insert from pCGP1805 was cloned in a "sense" orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (PGAP) in the expression vector pYE22m. TRP1 = Trp1 gene, IR1 = inverted repeat of 2 μm plasmid, TGAP = terminator sequence from the yeast glyceraldehyde-3-phosphate dehydrogenase gene. Restriction enzyme sites are also marked.

Figure 8 is a diagrammatic representation of the binary plasmid pCGP1867 (described in Example 8). The Htl cDNA insert (OGR-38) from pCGP1805 was cloned in a "sense" orientation behind the Mac promoter in the expression vector of pCGP293. Abbreviations are as follows: LB = left border; RB = right border; Gm = the gentamycin resistance gene; 35S = the promoter region from the Cauliflower Mosaic Virus 35S gene; nptII = the neomycin phosphotransferase II gene; tml3' = the terminator region from the tml gene of *Agrobacterium*; mas3' = the terminator region from the mannopine synthase gene of

Agrobacterium; ori pRi = a broad host range origin of replication from an *Agrobacterium rhizogenes* plasmid; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid. Restriction enzyme sites are also marked.

Figure 9 is a diagrammatic representation of the binary plasmid pCGP1810, preparation of which is described in Example 13. The KC-1 cDNA insert from pCGP1807 (see Example 12) was cloned in a "sense" orientation behind the Mac promoter in the expression vector of pCGP293. Abbreviations are as follows: LB = left border; RB = right border; Gm = the gentamycin resistance gene; 35S = the promoter region from the Cauliflower Mosaic Virus 35S gene; nptII = the neomycin phosphotransferase II gene; tml3' = the terminator region from the tml gene of *Agrobacterium*; mas3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; ori pRi = a broad host range origin of replication from a plasmid from *Agrobacterium rhizogenes*; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid. Restriction enzyme sites are also marked.

Figure 10 is a diagrammatic representation of the binary plasmid pCGP1813, construction of which is described in Example 14. The KC-1 cDNA insert from pCGP1807 (see Example 12) was cloned in a "sense" orientation between the mac promoter and mas terminator. The Mac: KC-1: mas expression cassette was subsequently cloned into the binary vector pWTT2132. Abbreviations are as follows: Tet= the tetracycline resistance gene; LB= left border; RB=right border, surB=the coding region and terminator sequence from the acetolactate synthase gene; 35S= the promoter region from the cauliflower mosaic virus 35S gene, mas3'=the terminator region from the mannopine synthase gene of *Agrobacterium;* pVS1 = a broad host range origin of replication from a plasmid from *Pseodomonas aeruginosa,* pACYCori= modified replicon from pACYC184 from *E. coli.* Restriction enzyme sites are also marked.

Figure 11 is a representation of an autoradiograph of a Southern blot probed with ^{32}P-labelled fragments of the Am3Ga differential display PCR fragment (as described in Example 16). Each lane contained a 10 μg sample of EcoRV-digested genomic DNA isolated from N8

(Eos$^+$), K16 (eos$^-$) or plants of an K16 x N8 F_2 population. Hybridizing bands were detected in the genomic DNA from cyanidin-producing plants (indicated with "+") (Lanes 1, 3, 4, 5, 6, 7, 9, 10, 12 and 15). No specific hybridization was observed in the genomic DNA samples from non-cyanidin-producing plants (indicated with "-") (Lanes 2, 8, 11, 13 and 14).

Figure 12 is a representation of an autoradiograph of an RNA blot probed with ^{32}P-labelled fragments of the Am3Ga differential display PCR fragment. Each lane contained a 10 μg sample of total RNA isolated from the flowers or leaves of plants of an N8 (Eos$^+$) x K16 (eos$^-$) F_2 population. A 1.8 kb transcript was detected in the K16 x N8 F_2 flowers that produced cyanidin (cyanidin +) (plants #1, #3, #4, #5 and #8). No transcript was detected in the K16 x N8 F_2 flowers that did not produce cyanidin (cyanidin -) (plants #6, #11, #12) or in a leaf sample (#13L) from an K16 x N8 F_2 plant that produced cyanidin in the flowers. Details are provided in Example 17.

Figure 13 is a diagrammatic representation of the binary plasmid pCGP250, construction of which is described in Example 20. The sdF3'H cDNA insert, containing the nucleotides 1 through to 1711 (SEQ ID NO:5) from pCGP246 (see Example 18), was cloned in a "sense" orientation behind the Mac promoter in the expression vector of pCGP293. Abbreviations are as follows: LB = left border; RB = right border; Gm = the gentamycin resistance gene; 35S = the promoter region from the Cauliflower Mosaic Virus 35S gene; nptII = the neomycin phosphotransferase II gene; tml3' = the terminator region from the tml gene of *Agrobacterium*; mas3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; ori pRi = a broad host range origin of replication from a plasmid from *Agrobacterium rhizogenes*; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid. Restriction enzyme sites are also marked.

Figure 14 is a diagrammatic representation of the binary plasmid pCGP231, construction of which is described in Example 20. The sdF3'H cDNA insert, containing the nucleotides 104 through to 1711 (SEQ ID NO:5) from pCGP246, was cloned in a "sense" orientation behind

the Mac promoter in the expression vector of pCGP293. Abbreviations are as follows: LB = left border; RB = right border; Gm = the gentamycin resistance gene; 35S = the promoter region from the Cauliflower Mosaic Virus 35S gene; nptII = the neomycin phosphotransferase II gene; tml3' = the terminator region from the tml gene of *Agrobacterium*; mas3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; ori pRi = a broad host range origin of replication from a plasmid from *Agrobacterium rhizogenes*; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid. Restriction enzyme sites are also marked.

Figure 15 is a diagrammatic representation of the binary plasmid pBI-Tt7-2. The 6.5 kb EcoRI/SalI Tt7 genomic fragment from E-5 was cloned into EcoRI/SalI-cut pBI101, replacing the resident GUS gene. The orientation of the Tt7 (F3'H) gene as indicated (5' to 3') was determined through DNA sequencing. Abbreviations are as follows: LB = left border; RB = right border; nos 5' = the promoter region from the nopaline synthase gene of *Agrobacterium;* nptII = the coding region of the neomycin phosphotransferase II gene; nos 3' = the terminator region from the nopaline synthase gene of *Agrobacterium;* nptI = the coding region of the neomycin phosphotransferase I gene. Restriction enzyme sites are also marked.

Figure 16 is a diagrammatic representation of the binary plasmid pCGP2166, construction of which is described in Example 26. The rose #34 cDNA insert from pCGP2158 (see Example 25) was cloned in a "sense" orientation behind the Mac promoter in the expression vector of pCGP293. Abbreviations are as follows: LB = left border; RB = right border; Gm = the gentamycin resistance gene; 35S = the promoter region from the Cauliflower Mosaic Virus 35S gene; nptII = the neomycin phosphotransferase II gene; tml3' = the terminator region from the tml gene of *Agrobacterium*; mas3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; ori pRi = a broad host range origin of replication from a plasmid from *Agrobacterium rhizogenes*; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid. Restriction enzyme sites are also marked.

Figure 17 is a diagrammatic representation of the binary plasmid pCGP2169 construction of which is described in Example 27. The rose #34 cDNA insert from pCGP2158 was cloned in a "sense" orientation between the CaMV35S promoter and the ocs terminator. The 35S: rose #34: ocs expression cassette was subsequently cloned into the binary vector pWTT2132. Abbreviations are as follows: Tet= the tetracycline resistance gene; LB= left border; RB=right border; surB=the boding region and terminator sequence from the acetolactate synthase gene; 35S=the promoter region from the cauliflowe mosaic virus 35S gene, ocs=terminator region from the octopine synthase gene from *Agrobacterium;* pVS1=a broad host range origin of replication from a plasmid from *Pseodomous aeruginosa,* pACYCori=modified replicon from pACYC184 from *E. coli.* Restriction enzyme sites are also marked.

Figure 18 is a diagrammatic representation of the binary plasmid pLN85, construction of which is described in Example 28. The chrysanthemum RM6i cDNA insert from pCHRM1 was cloned in "anti-sense" orientation behind the promoter from the Cauliflower Mosaic Virus 35S gene (35S). Other abbreviations are as follows: LB = left border; RB = right border; ocs3' = the terminator region from the octopine synthase gene of *Agrobacterium*; pnos:nptII:nos 3' = the expression cassette containing the promoter region from the nopaline synthase gene of *Agrobacterium*; the coding region of the neomycin phosphotransferase II gene and the terminator region from the nopaline synthase gene of *Agrobacterium*; oriT = origin of transfer of replication; trfA* = a trans-acting replication function; oriColE1 = a high copy origin of replication from a Colcinin E1 plasmid; Tn7SpR/StR = the spectinomycin and streptomycin resistance genes from transposon Tn7; oriVRK2 = a broad host range origin of replication from plasmid RK2. Restriction enzyme sites are also marked.

Figure 19 is a diagrammatic representation of the yeast expression plasmid pYTHT6, construction of which is described in Example 30. The THT6 cDNA insert from pTHT6 was cloned in a "sense" orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (PGAP) in the expression vector pYE22m. Abbreviations are as follows: TRP1 = Trp1 gene; IR1 = inverted repeat of 2 μm plasmid; TGAP = the terminator sequence from

the yeast glyceraldehyde-3-phosphate dehydrogenase gene. Restriction enzyme sites are also marked.

Amino acid abbreviations used throughout the specification are shown in Table 2, below.

TABLE 2

Amino acid abbreviations

Amino acid	3-letter	single-letter
L-alanine	Ala	A
L-arginine	Arg	R
L-asparagine	Asn	N
L-aspartic acid	Asp	D
L-cysteine	Cys	C
L-glutamine	Gln	Q
L-glutamic acid	Glu	E
L-glycine	Gly	G
L-histidine	His	H
L-isoleucine	Ile	I
L-leucine	Leu	L
L-lysine	Lys	K
L-methionine	Met	M
L-phenylalanine	Phe	F
L-proline	Pro	P
L-serine	Ser	S
L-threonine	Thr	T
L-tryptophan	Trp	W
L-tyrosine	Tyr	Y
L-valine	Val	V

Table 3 provides a summary of the SEQ ID NO's assigned to the sequences referred to herein:

TABLE 3

Sequence	Species	SEQ ID NO
cDNA insert of pCGP1805	Petunia	SEQ ID NO:1
corresponding amino acid sequence	Petunia	SEQ ID NO:2
cDNA insert of pCGP1807	Carnation	SEQ ID NO:3
corresponding amino acid sequence	Carnation	SEQ ID NO:4
cDNA insert of pCGP246	Snapdragon	SEQ ID NO:5
corresponding amino acid sequence	Snapdragon	SEQ ID NO:6
cDNA partial sequence	Arabidopsis	SEQ ID NO:7
corresponding amino acid sequence	Arabidopsis	SEQ ID NO:8
genomic sequence	Arabidopsis	SEQ ID NO:9
corresponding amino acid sequence for exon I	Arabidopsis	SEQ ID NO:10
corresponding amino acid sequence for exon II	Arabidopsis	SEQ ID NO:11
corresponding amino acid sequence for exon III	Arabidopsis	SEQ ID NO:12
corresponding amino acid sequence for exon IV	Arabidopsis	SEQ ID NO:13
cDNA insert of pCGP2158	Rose	SEQ ID NO:14
corresponding amino acid sequence	Rose	SEQ ID NO:15
cDNA insert of pCHRM1	Chrysanthemum	SEQ ID NO:16
corresponding amino acid sequence	Chrysanthemum	SEQ ID NO:17
THT cDNA sequence	Torenia	SEQ ID NO:18
corresponding amino acid sequence	Torenia	SEQ ID NO:19

MHT 85 cDNA sequence	Jap. Morning Glory	SEQ ID NO:20
corresponding amino acid sequence	Jap. Morning Glory	SEQ ID NO:21
GHT13 cDNA sequence	Gentian	SEQ ID NO:22
corresponding amino acid sequence	Gentian	SEQ ID NO:23
cDNA insert of pL3-6	Lisianthus	SEQ ID NO:24
corresponding amino acid sequence	Lisianthus	SEQ ID NO:25
cDNA sequence from WO 93/20206	Petunia	SEQ ID NO:26
oligonucleotide polyT-anchA		SEQ ID NO:27
oligonucleotide polyT-anchC		SEQ ID NO:28
oligonucleotide polyT-anchG		SEQ ID NO:29
conserved amino acid primer region		SEQ ID NO:30
corresponding oligonucleotide sequence		SEQ ID NO:31
conserved amino acid primer region		SEQ ID NO:32
corresponding oligonucleotide sequence		SEQ ID NO:33
oligonucleotide primer Pet Haem-New		SEQ ID NO:34
conserved amino acid primer region		SEQ ID NO:35
corresponding oligonucleotide sequence		SEQ ID NO:36
oligonucleotide Snapred Race A		SEQ ID NO:37
oligonucleotide Snapred Race C		SEQ ID NO:38
oligonucleotide poly-C Race		SEQ ID NO:39
oligonucleotide primer Pet Haem		SEQ ID NO:40

The disarmed microorganism *Agrobacterium tumefaciens* strain AGL0 separately containing the plasmids pCGP1867, pCGP1810 and pCGP231 were deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, New South Wales, 2037, Australia on 23 February, 1996 and were given Accession Numbers 96/10967, 96/10968 and 96/10969, respectively.

ISOLATION OF FLAVONOID 3'-HYDROXYLASE AND RELATED NUCLEIC ACID SEQUENCES

EXAMPLE 1-Plant Material

Petunia

The *Petunia hybrida* varieties used are presented in Table 4.

TABLE 4

Plant variety	Properties	Source/Reference
Old Glory Blue (OGB)	F_1 Hybrid	Ball Seed, USA
Old Glory Red (OGR)	F_1 Hybrid	Ball Seed, USA
V23	*An1, An2, An3, An4, An6, An8, An9, An10, ph1, Hf1, Hf2, ht1, Rt, po, Bl, Fl*	Wallroth *et al.* (1986) Doodeman *et al.* (1984)
R51	*An1, An2, An3, an4, An6, An8, An9, An10, An11, Ph1, hf1, hf2, Ht1, rt, Po, bl, fl*	Wallroth *et al.* (1986) Doodeman *et al.* (1984)
VR	V23 x R51 F_1 Hybrid	
SW63	*An1, An2, An3, an4, An6, An8, An9, An10, An11, Ph1, Ph2, Ph5, hf1, hf2, ht1, ht2, po, mf1, fl*	I.N.R.A., Dijon, Cedex France
Skr4	*An1, An2, An3, An4, An6, An11, hf1, hf2, ht1, Ph1, Ph2, Ph5, rt, Po, Mf1, Mf2, fl*	I.N.R.A., Dijon, Cedex France
Skr4 x SW63	F_1 Hybrid	

Plants were grown in specialised growth rooms with a 14 hour day length at a light intensity of 10,000 lux and a temperature of 22°C to 26°C.

Carnation

Flowers of *Dianthus caryophyllus* cv. Kortina Chanel were obtained from Van Wyk and Son

Flower Supply, Victoria.

Dianthus caryophyllus flowers were harvested at developmental stages defined as follows:

Stage 1: Closed bud, petals not visible.

Stage 2: Flower buds opening: tips of petals visible.

Stage 3: Tips of nearly all petals exposed. "Paint-brush stage".

Stage 4: Outer petals at 45° angle to stem.

Stage 5: Flower fully open.

Snapdragon

The *Antirrhinum majus* lines used were derived from the parental lines K16 (eos$^-$) and N8 (Eos$^+$). A strict correlation exists between F3'H activity and the Eos gene which is known to control the 3'-hydroxylation of flavones, flavonols and anthocyanins (Forkmann and Stotz, 1981). K16 is a homozygous recessive mutant lacking F3'H activity while N8 is wild type for F3'H activity. These lines are similar, though not isogenic. Both parental lines and the seed from a selfed (K16 x N8) F_1 plant were obtained from Dr C. Martin (John Innes Centre, Norwich, UK).

Arabidopsis

The *Arabidopsis thaliana* lines Columbia (Tt7), Landsberg erecta (Tt7) and NW88 (tt7) were obtained from the Nottingham Arabidopsis Stock Centre. Wild-type *A. thaliana* (Tt7) seeds have a characteristic brown colour. Seeds of tt7 mutants have pale brown seeds and the plants are characterized by a reduced anthocyanin content in leaves (Koornneef et al., 1982). Tt7 plants produce cyanidin while tt7 mutants accumulate pelargonidin, indicating that the Tt7 gene controls flavonoid 3'-hydroxylation.

Rose

Flowers of *Rosa hybrida* cv. Kardinal were obtained from Van Wyk and Son Flower Supply, Victoria.

Stages of *Rosa hybrida* flower development were defined as follows:

Stage 1: Unpigmented, tightly closed bud (10-12 mm high; 5 mm wide).

Stage 2: Pigmented, tightly closed bud (15 mm high ; 9 mm wide).

Stage 3: Pigmented, closed bud; sepals just beginning to open (20-25 mm high; 13-15 mm wide)

Stage 4: Flower bud beginning to open; petals heavily pigmented; sepals have separated (bud is 25-30 mm high and 18 mm wide).

Stage 5: Sepals completely unfolded; some curling. Petals are heavily pigmented and unfolding (bud is 30-33 mm high and 20 mm wide).

Chrysanthemum

Stages of *Chrysanthemum* flower development were defined as follows:

Stage 0: No visible flower bud.

Stage 1: Flower bud visible: florets completely covered by the bracts.

Stage 2: Flower buds opening: tips of florets visible.

Stage 3: Florets tightly overlapped.

Stage 4: Tips of nearly all florets exposed; outer florets opening but none horizontal.

Stage 5: Outer florets horizontal.

Stage 6: Flower approaching maturity.

EXAMPLE 2-Bacterial Strains

The *Escherichia coli* strains used were:

DH5α supE44, Δ(lacZYA-ArgF)U169, ø80lacZΔM15, hsdR17 (r_k-, m_k+), recA1, endA1, gyrA96, thi-1, relA1, deoR (Hanahan, 1983 and BRL, 1986).

XL1-Blue MRF' Δ(mcr A)183 , Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1,

recA1, gyrA96, relA1, lac[F' proAB, lacIqZΔM15, Tn10(Tet^r)]^c (Stratagene)

XL1-Blue supE44, hsdR17 (r_K-, m_K+), recA1, endA1, gyrA96, thi-1, relA1, lac[F' proAB, lacI^q, lacZΔM15, Tn10(tet^r)]

SOLR e14$^-$ (mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5(kan^r), uvrC,lac, gyrA96, thi-1, relA1, [F'proAB, lacI^{q}ZΔM15], Su$^-$ (non-suppressing) (Stratagene)

DH10 B(Zip) F$^-$mcrA, Δ(mrr-hsdRMS-mcrBC), ϕ80d lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara, leu)7697, galU, galKλ^-, rspL, nupG

Y1090r- ΔlacU169, (Δlon)?, araD139, strA, supF, mcrA, trpC22::Tn10(Tet^r) [pMC9 Amp^r, Tet^r], mcrB, hsdR

The disarmed *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991) was obtained from R. Ludwig (Department of Biology, University of California, Santa Cruz, USA).

The cloning vector pBluescript was obtained from Stratagene.

Transformation of the *E. coli* strain DH5α cells was performed according to the method of Inoue *et al.* (1990).

EXAMPLE 3-General methods

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 μCi of [α-^{32}P]-dCTP using an oligolabelling kit (Bresatec). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column.

DNA Sequence Analysis

DNA sequencing was performed using the PRISM™Ready Reaction Dye Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System9600) and run on an automated 373A DNA sequencer (Applied Biosystems).

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, 1988) or BLAST programs (Altschul *et al.*, 1990). Percentage sequence similarities were obtained using the LFASTA program (Pearson and Lipman, 1988). In all cases ktup values of 6 for nucleotide sequence comparisons and 2 for amino acid sequence comparisons were used, unless otherwise specified.

Multiple sequence alignments (ktup value of 2) were performed using the ClustalW program incorporated into the MacVector™6.0 application (Oxford Molecular Ltd.).

EXAMPLE 4- Isolation of a flavonoid 3'-hydroxylase (F3'H) cDNA clone corresponding to the Ht1 locus from *P. hybrida* cv. Old Glory Red

In order to isolate a cDNA clone that was linked to the Ht1 locus and that represented the flavonoid 3'-hydroxylase (F3'H) in the petunia flavonoid pathway, a petal cDNA library was prepared from RNA isolated from stages 1 to 3 of Old Glory Red (OGR) petunia flowers. OGR flowers contain cyanidin based pigments and have high levels of flavonoid 3'-hydroxylase activity. The OGR cDNA library was screened with a mixture of ^{32}P-labelled fragments isolated from three cytochrome P450 cDNA clones known to be involved in the flavonoid pathway and from one cytochrome P450 cDNA clone (651) that had flavonoid 3'-hydroxylase activity in yeast. These included a petunia cDNA clone representing the cinnamate-4-hydroxylase (C4H) and two petunia cDNA clones (coded by the Hf1 and Hf2 loci) representing flavonoid 3' 5'-hydroxylase (F3' 5'H) (Holton *et al.*, 1993).

Construction of Petunia cv. OGR cDNA library

Total RNA was isolated from the petal tissue of *P. hybrida* cv OGR stage 1 to 3 flowers using the method of Turpen and Griffith (1986). Poly(A)$^+$ RNA was selected from the total RNA, using oligotex-dT™ (Qiagen).

A ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene) was used to construct a directional petal cDNA library in λZAP using 5 μg of poly(A)+ RNA isolated from stages 1 to 3 of OGR as template. The total number of recombinants obtained was 2.46×10^6.

After transfecting XL1-Blue MRF' cells, the packaged cDNA mixture was plated at 50,000 pfu per 15 cm diameter plate. The plates were incubated at 37°C for 8 hours, and the phage were eluted in 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 8.0, 0.01% (w/v) gelatin (Phage Storage Buffer (PSB)) (Sambrook *et al.*, 1989). Chloroform was added and the phage stored at 4°C as an amplified library.

100,000 pfu of the amplified library were plated onto NZY plates (Sambrook *et al.*, 1989) at a density of 10,000 pfu per 15 cm plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After incubation at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Isolation of probes

F3'5'H probes

The two flavonoid 3', 5' hydroxylases corresponding to the Hf1 or Hf2 loci isolated as described in Holton *et al.* (1993) and US Patent Number 5,349,125, were used in the screening process.

C4H cDNA clones from petunia

A number of cytochrome P450 cDNA clones were isolated in the screening process used to

isolate the two flavonoid 3', 5' hydroxylase cDNA clones corresponding to the Hf1 or Hf2 loci (Holton *et al.*, 1993; US Patent Number 5,349,125). One of these cDNA clones (F1) (contained in pCGP161) (Figure 2) was identified as representing a cinnamate 4-hydroxylase (C4H), based on sequence identity with a previously-characterised C4H clone from mung bean (Mizutani *et al.*, 1993). Sequence data was generated from 295 nucleotides at the 5' end of the petunia F1 cDNA clone. There was 83.1% similarity with the mung bean C4H clone over the 295 nucleotides sequenced and 93.9% similarity over the predicted amino acid sequence.

651 cDNA clone

The isolation and identification of the 651 cDNA clone contained in pCGP619 (Figure 5) was described in the International Patent Application, having publication number W093/20206. A protein extract of yeast containing the 651 cDNA clone under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter of pYE22m (Tanaka *et al.*, 1988) exhibited F3'H activity.

Screening of OGR Library

Prior to hybridization, the duplicate plaque lifts were washed in prewashing solution (50 mM Tris-HCl pH7.5, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) sarcosine) at 65°C for 30 minutes; stripped in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% (w/v) SDS.

The lifts from the OGR cDNA library were screened with ^{32}P-labelled fragments of (1) a 0.7 kb EcoRI/XhoI fragment from pCGP161 containing the C4H cDNA clone (Figure 2), (2) a 1.6 kb BspHI/EspI fragment from pCGP602 containing the Hf1 cDNA clone (Figure 3), (3) a 1.3 kb EcoRI/XhoI fragment and a 0.5 kb XhoI fragment from pCGP175 containing the coding region of the Hf2 cDNA clone (Figure 4) and (4) a 1.8 kb EcoRI/XhoI fragment pCGP619 containing the 651 cDNA clone (Figure 5).

Hybridization conditions included a prehybridization step in 10% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragments (each at 1x10^{6}cpm/mL) were then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% (w/v) SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Two hundred and thirty strongly hybridizing plaques were picked into PSB. Of these, 39 were rescreened to isolate purified plaques, using the hybridization conditions as described for the initial screening of the cDNA library. The plasmids contained in the λZAP bacteriophage vector were rescued and sequence data was generated from the 3' and 5' ends of the cDNA inserts. Based on sequence homology, 27 of the 39 were identical to the petunia cinnamate 4-hydroxylase cDNA clone, 2 of the 39 were identical to the Hf1 cDNA clone and 7 of the 39 did not represent cytochrome P450s. The remaining 3 cDNA clones (designated as OGR-27, OGR-38, OGR-39) represented "new" cytochrome P450s, compared to the cytochrome P450 clones used in the screening procedure, and were further characterised.

EXAMPLE 5 -Restriction Fragment Length Polymorphism (RFLP) analysis

There are two genetic loci in *P. hybrida*, Ht1 and Ht2, that control flavonoid 3'-hydroxylase activity (Tabak *et al.*, 1978; Wiering and de Vlaming, 1984). Ht1 is expressed in both the limb and the tube of *P. hybrida* flowers and gives rise to higher levels of F3'H activity than does Ht2 which is only expressed in the tube. The F3'H is able to convert dihydrokaempferol and naringenin to dihydroquercetin and eriodictyol, respectively. In a flower producing delphinidin-based pigments, F3'H activity is masked by the F3'5'H activity. Therefore, the F3'H/F3'5'H assay (Stotz and Forkmann, 1982) is useless in determining the presence or absence of F3'H activity. The enzyme flavonol synthase is able to convert dihydrokaempferol to kaempferol and dihydroquercetin to quercetin (Figure 1a). Myricetin, the 3', 5' hydroxylated flavonol, is produced at low levels in petunia flowers.

Therefore, analysing the flowers for the 3' hydroxylated flavonol, quercetin, infers the presence of F3'H activity.

Restriction Fragment Length Polymorphism (RFLP) analysis of DNA isolated from individual plants in a VR (Ht1/ht1) x V23 (ht1/ht1) backcross was used to determine which, if any, of the cDNA clones representing P450s were linked to the Ht1 locus. Northern analysis of RNA isolated from these plants was also used to detect the presence or absence of a transcript in these lines.

Flowers from a VR (Ht1/ht1) x V23 (ht1/ht1) backcross population were analysed for the presence of the flavonols, kaempferol and quercetin. VR (Ht1/ht1) flowers accumulate quercetin and low levels of kaempferol while V23 (ht1/ht1) flowers accumulate kaempferol but little or no quercetin. Individual plants from the VR (Ht1/ht1) x V23 (ht1/ht1) backcross were designated as VR-like (Ht1/ht1), if a substantial level of quercetin was detected in the flower extracts, and V23-like (ht1/ht1), if little or no quercetin but substantial levels of kaempferol were detected in the flower extracts (see Figure 6).

Isolation of Genomic DNA

DNA was isolated from leaf tissue essentially as described by Dellaporta *et al.*, (1983). The DNA preparations were further purified by CsCl buoyant density centrifugation (Sambrook *et al.*, 1989).

Southern blots

The genomic DNA (10 μg) was digested for 16 hours with 60 units of EcoRI and electrophoresed through a 0.7% (w/v) agarose gel in a running buffer of TAE (40 mM Tris-acetate, 50 mM EDTA). The DNA was then denatured in denaturing solution (1.5 M NaCl/0.5 M NaOH) for 1 to 1.5 hours, neutralized in 0.5 M Tris-HCl (pH 7.5)/ 1.5 M NaCl for 2 to 3 hours and then transferred to a Hybond N (Amersham) filter in 20 x SSC.

RNA blots

Total RNA was isolated from the petal tissue of *P. hybrida* cv OGR stage 1 to 3 flowers using the method of Turpen and Griffith (1986).

RNA samples were electrophoresed through 2.2 M formaldehyde/1.2% (w/v) agarose gels using running buffer containing 40 mM morpholinopropanesulphonic acid (pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA (pH 8.0). The RNA was transferred to Hybond-N filters (Amersham) as described by the manufacturer.

Hybridization and washing conditions

Southern and RNA blots were probed with ^{32}P-labelled cDNA fragment (10^8 cpm/μg, 2 x 10^6 cpm/mL). Prehybridizations (1 hour at 42°C) and hybridizations (16 hours at 42°C) were carried out in 50% (v/v) formamide, 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate. Filters were washed in 2 x SSC, 1% (w/v) SDS at 65°C for 1 to 2 hours and then 0.2 x SSC, 1% (w/v) SDS at 65°C for 0.5 to 1 hour. Filters were exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

RFLP and Northern analysis of the cytochrome P450 fragments

RFLP analysis was used to investigate linkage of the genes corresponding to the OGR-27, OGR-38 and OGR-39 cDNA clones to the Ht1 locus.

^{32}P-labelled fragments of OGR-27, OGR-38 and OGR-39 cDNA clones were used to probe RNA blots and Southern blots of genomic DNA isolated from individual plants in the VR x V23 backcross population. Analysis of EcoRI digested genomic DNA isolated from a VR x V23 backcross population revealed a RFLP for the OGR-38 probe which was linked to Ht1. Furthermore, a much reduced level of transcript was detected in the V23-like lines, when compared with the high levels of transcript detected in VR-like lines (Figure 6).

The data provided strong evidence that the OGR-38 cDNA clone, contained in plasmid pCGP1805, corresponded to the Ht1 locus and represented a F3'H.

RFLP analysis of a V23 x R51 F_2 backcross

RFLP analysis was used to investigate linkage of the gene corresponding to the OGR-38 cDNA to known genetic loci.

The RFLP linkage analysis was performed using a Macintosh version 2.0 of the MapMaker mapping program (Du Pont) (Lander *et al*, 1987). A LOD score of 3.0 was used for the linkage threshold.

Analysis of EcoRI or XbaI digested genomic DNA isolated from a V23 x R51 F_2 population revealed a RFLP for the OGR-38 probe which was linked to PAc4. PAc4, a petunia actin cDNA clone (Baird and Meagher, 1987), is a molecular marker for chromosome III and is linked to the Htl locus (McLean *et al.*, 1990). There was co-segregation of the OGR-38 and PAc4 RFLPs for 36 out of 44 V23 x R51 F_2 plants. This represents a recombination frequency of 8% which is similar to a reported recombination frequency of 16% between the Ht1 locus and PAc4 (Cornu *et al.*, 1990).

Further characterisation of OGR-38

The developmental expression profiles in OGR petals, as well as in other OGR tissues, were determined by using the ^{32}P-labelled fragments of the OGR-38 cDNA insert as a probe against an RNA blot containing 20μg of total RNA isolated from each of the five petunia OGR petal developmental stages as well as from leaves, sepals, roots, stems, peduncles, ovaries, anthers and styles. The OGR-38 probe hybridized with a 1.8kb transcript that peaked at the younger stages of 1 to 3 of flower development. The OGR-38 hybridizing transcript was most abundant in the petals and ovaries and was also detected in the sepals, peduncles and anthers of the OGR plant. A low level of transcript was also detected in the stems. Under the conditions used, no hybridizing transcript was detected by Northern analysis of total RNA isolated from leaf, style or roots.

EXAMPLE 6- Complete sequence of OGR-38

The complete sequence of the OGR-38 cDNA clone (SEQ ID NO:1) was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989). The sequence contained an open reading frame of 1536 bases which encodes a putative polypeptide of 512 amino acids.

The nucleotide and predicted amino acid sequences of OGR-38 (SEQ ID NO:1 and SEQ ID NO:2) were compared with those of the cytochrome P450 probes used in the screening process and with other petunia cytochrome P450 sequences (US Patent Number 5,349,125) using an lfasta alignment (Pearson and Lipman, 1988). The nucleotide sequence of OGR-38 was most similar to the nucleic acid sequence of the flavonoid 3' 5'-hydroxylases representing Hf1 and Hf2 loci from *P. hybrida* (Holton *et al.*, 1993). The Hf1 clone showed 59.6% similarity to the OGR-38 cDNA clone, over 1471 nucleotides, and 49.9% similarity, over 513 amino acids, while the Hf2 clone showed 59.1% similarity to the OGR-38 cDNA clone, over 1481 nucleotides, and 49.0% similarity, over 511 amino acids.

EXAMPLE 7- The F3'H assay of the Ht1 cDNA clone (OGR-38) expressed in yeast

Construction of pCGP1646

The plasmid pCGP1646 (Figure 7) was constructed by cloning the OGR-38 cDNA insert from pCGP1805 in a "sense" orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter of pYE22m (Tanaka *et al.*, 1988).

The plasmid pCGP1805 was linearised by digestion with Asp718. The overhanging 5' ends were "filled in" using DNA polymerase (Klenow fragment) according to standard protocols (Sambrook *et al.*, 1989). The 1.8 kb OGR-38 cDNA fragment was released upon digestion with SmaI. The cDNA fragment was isolated and purified using the Bresaclean kit (Bresatec) and ligated with blunted EcoRI ends of pYE22m. The plasmid pYE22m had been digested with EcoRI and the overhanging 5' ends were removed using DNA polymerase

(Klenow fragment) according to standard protocols (Sambrook *et al.*, 1989). The ligation was carried with the Amersham Ligation kit using 100ng of the 1.8 kb OGR-38 fragment and 150ng of the prepared yeast vector, pYE22m. Correct insertion of the insert in pYE22m was established by XhoI/SalI restriction enzyme analysis of the plasmid DNA isolated from ampicillin-resistant transformants.

Yeast transformation

The yeast strain G-1315 (Mat α, trp1) (Ashikari *et al.*, 1989) was transformed with pCGP1646 according to Ito *et al.* (1983). The transformants were selected by their ability to restore G-1315 to tryptophan prototrophy.

Preparation of yeast extracts for assay of F3'H activity

A single isolate of G-1315/pCGP1646 was used to inoculate 50 mL of Modified Burkholder's medium (20.0g/L dextrose, 2.0g/L L-asparagine, 1.5g/L KH_2PO_4, 0.5g/L $MgSO_4.7H_2O$, 0.33g/L $CaCl_2$, 2g/L $(NH_4)_2SO_4$, 0.1 mg/L KI, 0.92g/L $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1g/L nitrilotriacetic acid, 0.99 mg/L $FeSO_4.7H_2O$, 1.25 mg/L EDTA, 5.47 mg/L $ZnSO_4.7H_2O$, 2.5 mg/L $FeSO_4.7H_2O$, 0.77 mg/L $MnSO_4.7H_2O$, 0.196 mg/L $CuSO_4.5H_2O$, 0.124 mg/L $Co(NH_4)_2(SO_4)_2.6H_2O$, 0.088 mg/L $Na_2B_4O_7.10H_2O$, 0.2 mg/L thiamine, 0.2 mg/L pyridoxine, 0.2 mg/L nicotinic acid, 0.2 mg/L pantothenate, 0.002 mg/L biotin, 10 mg/L inositol) which was subsequently incubated until the value at OD_{600} was 1.8 at 30°C. Cells were collected by centrifugation and resuspended in Buffer 1 [10 mM Tris-HCl buffer (pH 7.5) containing 2 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mg yeast lytic enzyme/mL]. Following incubation for 1 hour at 30°C with gentle shaking, the cells were pelleted by centrifugation and washed in ice cold Buffer 2 [10 mM Tris-HCl (pH7.5) containing 0.65 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF]. The cells were then resuspended in Buffer 2 and sonicated using six 15-second bursts with a Branson Sonifier 250 at duty cycle 30% and output control 10%. The sonicated suspension was centrifuged at 10,000 rpm for 30 minutes and the supernatant was centrifuged at 13,000 rpm for 90 minutes. The microsomal pellet was resuspended in assay buffer (100 mM potassium

phosphate (pH 8), 1 mM EDTA, 20 mM 2-mercaptoethanol) and 100 μL was assayed for activity.

F3'H Assay

F3'H enzyme activity was measured using a modified version of the method described by Stotz and Forkmann (1982). The assay reaction mixture typically contained 100 μL of yeast extract, 5 μL of 50 mM NADPH in assay buffer (100 mM potassium phosphate (pH8.0), 1 mM EDTA and 20 mM 2-mercaptoethanol) and 10 μCi of [^{3}H]- naringenin and was made up to a final volume of 210 μL with the assay buffer. Following incubation at 23°C for 2-16 hours, the reaction mixture was extracted with 0.5 mL of ethylacetate. The ethylacetate phase was dried under vacuum and then resuspended in 10 μL of ethylacetate. The tritiated flavonoid molecules were separated on cellulose thin layer plates (Merck Art 5577, Germany) using a chloroform: acetic acid: water (10:9:1 v/v) solvent system. The reaction products were localised by autoradiography and identified by comparison to non-radioactive naringenin and eriodictyol standards which were run alongside the reaction products and visualised under UV light.

F3'H activity was detected in extracts of G1315/pCGP1646, but not in extracts of non-transgenic yeast. From this it was concluded that the cDNA insert from pCGP1805 (OGR-38), which was linked to the Ht1 locus, encoded a F3'H.

EXAMPLE 8- Transient expression of the Ht1 cDNA clone (OGR-38) in plants

Construction of pCGP1867

Plasmid pCGP1867 (Figure 8) was constructed by cloning the cDNA insert from pCGP1805 in a "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP293 (Brugliera *et al.*, 1994). The plasmid pCGP1805 was digested with XbaI and KpnI to release the cDNA insert. The cDNA fragment was isolated and purified using the Bresaclean kit (Bresatec) and ligated with XbaI/KpnI ends of the pCGP293 binary vector. The ligation was carried out using the Amersham ligation kit. Correct insertion of the fragment in

pCGP1867 was established by XbaI/KpnI restriction enzyme analysis of DNA isolated from gentamycin-resistant transformants.

Transient expression of the Ht1 cDNA clone (OGR-38) in petunia petals

In order to rapidly determine whether the OGR-38 cDNA fragment in pCGP1867 represented a functional F3'H in plants, a transient expression study was established. Petals of the mutant *P. hybrida* line Skr4 x SW63 were bombarded with gold particles (1μm diameter) coated with pCGP1867 DNA.

Gold microcarriers were prewashed 3 times in 100% ethanol and resuspended in sterile water. For each shot, 1 μg of pCGP1867 DNA, 0.5 mg of gold microcarriers, 10 μL of 2.5 M $CaCl_2$ and 2 μL of 100 mM spermidine (free base) were mixed by vortexing for 2 minutes. The DNA coated gold particles were pelleted by centrifugation, washed twice with 100% ethanol and finally resuspended in 10 μL of 100% ethanol. The suspension was placed directly on the centre of the macrocarrier and allowed to dry.

Stages 1 and 2 of Skr4 x SW63 flowers were cut vertically into halves and partially embedded in MS solid media (3% (w/v) sucrose, 100 mg/L myo-inositol, 1xMS salts, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, 0.5 mg/L nicotinic acid and 2 mg/L glycine). The petals were placed so that the inside of the flower buds were facing upwards. A Biolistic PDS-1000/He System (Bio-Rad), using a Helium gas pressure of 900psi and a chamber vacuum of 28 inches of mercury, was used to project the gold microcarriers into the petal tissue. After 6-12 hours under lights in a controlled plant growth room at 22°C, red anthocyanin spots were observed on the upper epidermal layer of the petal tissue bombarded with pCGP1867-coated particles. No coloured spots were observed in control petal bombarded with gold particles alone. These results indicated that the OGR-38 cDNA clone under the control of the Mac promoter was functional, at least transiently, in petal tissue.

EXAMPLE 9- Stable expression of the Ht1 cDNA clone (OGR-38) in petunia petals-Complementation of a ht1/ht1 petunia cultivar

***A. tumefaciens* transformations**

The plasmid pCGP1867 (Figure 8) was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL MG/L (Garfinkel and Nester, 1980) culture and growing for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% (v/v) 100 mM $CaCl_2$/15% (v/v) glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells were then mixed with 1 mL of LB (Sambrook *et al.*, 1989) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying pCGP1867 were selected on LB agar plates containing 10 μg/mL gentamycin. The presence of pCGP1867 was confirmed by Southern analysis of DNA isolated from the gentamycin-resistant transformants.

Petunia transformations

(a) Plant Material

Leaf tissue from mature plants of *P. hybrida* cv Skr4 x SW63 was treated in 1.25% (w/v) sodium hypochlorite for 2 minutes and then rinsed three times in sterile water. The leaf tissue was then cut into 25 mm^2 squares and precultured on MS media (Murashige and Skoog, 1962) supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 hours.

(b) Co-cultivation of *Agrobacterium* and Petunia Tissue

A. tumefaciens strain AGL0 containing the binary vector pCGP1867 (Figure 11) was maintained at 4°C on MG/L agar plates with 100 mg/L gentamycin. A single colony was grown overnight in liquid medium containing 1% (w/v) Bacto-peptone, 0.5% (w/v) Bacto-

yeast extract and 1% (w/v) NaCl. A final concentration of 5 x 10^8 cells/mL was prepared the next day by dilution in liquid MS medium containing B5 vitamins (Gamborg *et al.*, 1968) and 3% (w/v) sucrose (BPM). The leaf discs were dipped for 2 minutes into BPM containing AGL0/pCGP1867. The leaf discs were then blotted dry and placed on co-cultivation media for 4 days. The co-cultivation medium consisted of SH medium (Schenk and Hildebrandt, 1972) supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-D and included a feeder layer of tobacco cell suspension spread over the co-cultivation medium with a filter paper placed on top of the tobacco cell suspension.

(c) Recovery of transgenic petunia plants

After co-cultivation, the leaf discs were transferred to selection medium (MS medium supplemented with 3% (w/v) sucrose, α-benzylaminopurine (BAP) 2 mg/L, 0.5 mg/L α-naphthalene acetic acid (NAA), kanamycin 300 mg/L, 350 mg/L cefotaxime and 0.3% (w/v) Gelrite Gellan Gum (Schweizerhall)). Regenerating explants were transferred to fresh selection medium after 4 weeks. Adventitious shoots which survived the kanamycin selection were isolated and transferred to BPM containing 100 mg/L kanamycin and 200 mg/L cefotaxime for root induction. All cultures were maintained under a 16 hour photoperiod (60 μmol. m-2, s-1 cool white fluorescent light) at 23± 2°C. When roots reached 2-3 cm in length the transgenic petunia plantlets were transferred to autoclaved Debco 51410/2 potting mix in 8 cm tubes. After 4 weeks, plants were replanted into 15 cm pots, using the same potting mix, and maintained at 23°C under a 14 hour photoperiod (300 μmol. m-2, s-1 mercury halide light).

EXAMPLE 10 -Transgenic plant phenotype analysis

pCGP1867 in Skr4 x SW63

Table 5 shows the various petal and pollen colour phenotypes obtained with Skr4 x SW63 plants transformed with the pCGP1867 plasmid. The transgenic plants #593A, 590A, 571A, 589A, 592A and 591A produced flowers with altered petal colour. Moreover, the anthers and pollen of the flowers from plants #593A, 590A, 589A, 592A and 591A were pink,

compared with those of the control Skr4 x SW63 plant, which were white. The change in anther and pollen colour, observed on introduction of plasmid pCGP1867 into Skr4 x SW63 petunia plants, was an unanticipated outcome. The colour codes are taken from the Royal Horticultural Society's Colour Chart (RHSCC). They provide an alternative means by which to describe the colour phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colours and should not be regarded as limiting the possible colours which may be obtained.

TABLE 5

Summary of petal, anther and pollen colours obtained in Skr4 xSW63 plants transformed with pCGP1867

Accession Number	Petal Limb Colour	RHSCC Code (petal limb)	Anther & Pollen Colour
Skr4 x SW63 control (594A)	very pale lilac	69B/73D	white
593A	dark pink	67B	pink
590A	dark pink and pink sectors	sectored 67B and 73A	pink
571A	pink	68A and B	pink
589A	dark pink	68A	pink
592A	pink and light pink sectors	68A and 68B	light pink
591A	dark pink	68A	pink
570A	very pale lilac	69B/73D	white

The expression of the introduced Ht1 cDNA in the Skr4 x SW63 hybrid had a marked effect on flower colour. The stamen tissue of the non-transgenic control is white, whereas the same tissue in most of the transgenic plants was pink. In addition, expression of the Ht1 cDNA in the Skr4 x SW63 hybrid conferred a dark pink hue to the corolla, which is normally very pale lilac.

EXAMPLE 11- Analysis of products

The anthocyanidins and flavonols produced in the petals and stamens (included the pollen, anthers and filaments) of the Skr4 x SW63 plants transformed with pCGP1867 were analysed by TLC.

Extraction of anthocyanins and flavonols

Prior to TLC analysis, the anthocyanin and flavonol molecules present in petal and stamen extracts were acid hydrolysed to remove glycosyl moieties from the anthocyanidin or flavonol core. Anthocyanidin and flavonol standards were used to help identify the compounds present in the floral extracts.

Anthocyanins and flavonols were extracted and hydrolysed by boiling between 100 to 200 mg of petal limbs, or five stamens, in 1 mL of 2 M hydrochloric acid for 30 minutes. The hydrolysed anthocyanins and flavonols were extracted with 200 μL of iso-amylalcohol. This mixture was then dried down under vacuum and resuspended in a smaller volume of methanol/1% (v/v) HCl. The volume of methanol/1% (v/v) HCl used was based on the initial fresh weight of the petal so that the relative levels of flavonoids in the petals could be estimated. Extracts from the stamens were resuspended in 1 μL of methanol/1% (v/v) HCl. A 1 μL aliquot of the extracts from the pCGP1867 in Skr4 x SW63 petals and stamens was spotted onto a TLC plate.

TLC analysis of floral extracts

Acid-hydrolysed floral extracts were run in a Forestal solvent system (HOAc:water:HCl; 30: 10: 3) (Markham, 1982). Table 6 shows the results of the TLC analysis of the anthocyanidins and flavonols present in some of the flowers and stamens of the transgenic Skr4 x SW63 petunia plants transformed with pCGP1867. Indicative relative amounts of the flavonols and anthocyanidins (designated with a "+" to "+++") were estimated by comparing the intensities of the spots observed on the TLC plate.

TABLE 6

Relative levels of anthocyanidins and flavonols detected in the petal limbs and stamens of Skr4 x SW63 plants transformed with pCGP1867.

Acc#	Petal Colour	Anthocyanidins			Flavonols	
		Malvidin	Cyanidin	Peonidin	Kaempferol	Quercetin
Skr4 x SW63 control petal limb	pale lilac	+/-	-	-	+	-
593A petal limb	dark pink	-	+	+++	-	++
571A petal limb	pink	-	+	+	-	+
589A petal limb	dark pink	-	+	++	-	++
570A petal limb	pale lilac	+/-	-	-	+	-
Skr4 x SW63 control stamens	white	-	-	-	+++	+
593A stamens	pink	-	-	++	-	+++

Introduction of the Ht1 cDNA clone into Skr4 x SW63 led to production of the 3'-hydroxylated flavonoids, quercetin, peonidin and some cyanidin in the petals. Peonidin is the methylated derivative of cyanidin (Figures 1a and 1b). Only kaempferol and a small amount of malvidin were detected in the non-transgenic Skr4 x SW63 control (Table 6). Although Skr4 x SW63 is homozygous recessive for both the Hf1 and Hf2 genes, these mutations do not completely block production of F3'5'H (see US Patent Number 5,349,125) and low levels of malvidin are produced to give the petal limb a pale lilac colour.

The stamens with the pink pollen and anthers produced by the transgenic plant #593A contained peonidin and quercetin, while the non-transgenic Skr4 x SW63 control with white pollen and anthers contained kaempferol and a low level of quercetin (Table 6).

The accumulation of the 3'-hydroxylated anthocyanidin, peonidin, in the petals and stamens of the transgenic Skr4 x SW63/pCGP1867 plants correlated with the pink and dark pink colours observed in the petals, anthers and pollen of the same plants.

Co-suppression of F3'H activity

The plasmid pCGP1867 was also introduced into *P. hybrida* cv. Old Glory Red (Ht1) in order to reduce the level of F3'H activity.

Petunia transformations were carried out as described in Example 9, above.

Two out of 38 trangenic plants produced flowers with an altered phenotype. OGR normally produces deep red flowers (RHSCC#46B). The two transgenic plants with altered floral colour produced flowers with a light pink or light red hue (RHSCC#54B and #53C).

Northern analysis on RNA isolated from flowers produced by four transgenic plants (the two transgenics with an altered phenotype and two transgenics with the usual deep red flowers) was performed to examine the level of OGR-38 transcripts. Ten micrograms of total petal RNA was separated on a 1.2% (w/v) agarose/formaldehyde gel (Sambrook *et al.* 1989) and transferred to HybondN nylon membrane (Amersham), as described previously. Petal RNA from a non-transformed OGR flower was also included as a control. ^{32}P-labelled fragments of the OGR-38 cDNA inserts were used to probe the RNA blot.

The OGR-38 probe detected transcripts of approximately 2.4 kb and 1.8 kb in the flowers of the transgenic plants. However, the level of both transcripts detected in the light pink and light red flowers was considerably lower than that detected in the deep red transgenic flowers. The endogenous 1.8 kb transcript was also detected in RNA from the non-transformed OGR flowers. In order to confirm that the 2.4kb transcript was from the introduced OGR-38 transgene, ^{32}P-labelled fragments of the *mas* terminator region were used to probe the same RNA blot. The *mas* probe detected the 2.4 kb transcript, suggesting that at least this transcript was derived from the introduced OGR-38 transgene.

Analysis of anthocyanin levels

The levels of anthocyanins in the control flowers and in the light pink transgenic flower were measured by spectrophotometric analysis.

Extraction of anthocyanins and flavonols

Anthocyanins and flavonols were extracted from petal limbs by incubating 200 to 300mg of petal limb in 2mL of methanol/1% (v/v) HCl for 16 hours at 4°C. Fifty μL of this solution was then added to 950μL of methanol/1% (v/v) HCl and the absorbance of the diluted solution at 530nm was determined. The anthocyanin level in nmoles per gram was determined using the formula: [(Abs (530 nm)/34,000) x volume of extraction buffer x dilution factor x 10^6] / weight in grams.

The light pink flower was found to contain approximately 915 nmoles of anthocyanin per gram of petal limb tissue whilst the control flower contained around 4000nmoles/gram.

These data suggest that introduction of the petunia F3'H (OGR-38) cDNA clone in a sense orientation into OGR plants leads to "co-suppression" (i.e. reduction) of both the endogenous and the transgenic F3'H transcripts. A correlation was observed between lighter flower colours, reduced anthocyanin production and reduced F3'H transcript level.

EXAMPLE 12- Isolation of a F3'H cDNA clone from *Dianthus caryophyllus*

In order to isolate a *Dianthus caryophylluss* (carnation) F3'H cDNA clone, the petunia Ht1-linked F3'H cDNA clone (OGR-38), contained in pCGP1805 (described above), was used to screen a Carnation cv. Kortina Chanel petal cDNA library, under low stringency conditions.

Construction of Carnation cv. Kortina Chanel cDNA library

Twenty micrograms of total RNA isolated (as described previously) from stages 1, 2 and 3 of Kortina Chanel flowers was reverse transcribed in a 50 μL volume containing 1 x Superscript™ reaction buffer, 10 mM dithiothreitol (DTT), 500 μM dATP, 500 μM dGTP, 500 μM dTTP, 500 μM 5-methyl-dCTP, 2.8 μg Primer-Linker oligo from ZAP-cDNA Gigapack III Gold cloning kit (Stratagene) and 2 μL Superscript™ reverse transcriptase (BRL). The reaction mix was incubated at 37°C for 60 minutes, then placed on ice. A

ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene) was used to complete the library construction. The total number of recombinants was 2.4 x 10^6.

A total of 200,000 pfu of the packaged cDNA was plated at 10,000 pfu per 15 cm diameter plate after transfecting XL1-Blue MRF' cells. The plates were incubated at 37°C for 8 hours, then stored overnight at 4°C. Duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Screening of Kortina Chanel petal cDNA library for a F3'H cDNA clone

Prior to hybridization, the duplicate plaque lifts were treated as described previously. The duplicate lifts from the Kortina Chanel petal cDNA library were screened with ^{32}P-labelled fragments of the 1.8 kb EcoRI/XhoI insert from pCGP1805. Low stringency conditions, as described for the screening of the petunia OGR cDNA library, were used.

One strongly-hybridizing plaque was picked into PSB and rescreened as detailed above to isolate purified plaques. The plasmid contained in the lZAP bacteriophage vector was rescued and named pCGP1807.

The KC-1 cDNA insert contained in pCGP1807 was released upon digestion with EcoRI/XhoI and is around 2 kb. The complete sequence of the KC-1 cDNA clone was determined by compilation of sequence from subclones of the KC-1 cDNA insert. (Partial sequence covering 458 nucleotides had previously been generated from a 800 bp KpnI fragment covering the 3' region of KC-1 which was subcloned into pBluescript to give pCGP1808.) The complete sequence (SEQ ID NO:3) contained an open reading frame of 1508 bases which encodes a putative polypeptide of 500 amino acids (SEQ ID NO:4).

The nucleotide and predicted amino acid sequences of the carnation KC-1 cDNA clone were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2). The sequences of the carnation KC-1 cDNA clone (SEQ ID NO:3 and 4) showed 67.3% similarity, over 1555 nucleotides, and 71.5 % similarity, over 488 amino acids, to

that of the petunia OGR-38 F3'H cDNA clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 13- Stable expression of the carnation F3'H cDNA (KC-1) clone in petunia petals- Complementation of a ht1/ht1 petunia cultivar

Preparation of pCGP1810

Plasmid pCGP1810 (Figure 9) was constructed by cloning the cDNA insert from pCGP1807 in a "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP90 (US Patent Number 5,349,125), a pCGP293 based construct (Brugliera *et al.*, 1994). The plasmid pCGP1807 was digested with BamHI and ApaI to release the KC-1 cDNA insert. The cDNA fragment was isolated and purified using the Bresaclean kit (Bresatec). The pCGP90 binary vector was digested with BamHI and ApaI to release the linearised vector and the Hf1 cDNA insert. The linearised vector was isolated and purified using the Bresaclean kit (Bresatec) and ligated with BamHI/ApaI ends of the KC-1 cDNA clone. The ligation was carried out using the Amersham ligation. Correct insertion of the insert in pCGP1810 was established by BamHI/ApaI restriction enzyme analysis of DNA isolated from gentamycin-resistant transformants.

The binary vector pCGP1810 was introduced into *A. tumefaciens* strain AGL0 cells, as described in Example 9. The pCGP1810/AGL0 cells were subsequently used to transform Skr4 x SW63 petunia plants (also described in Example 9), to test for stable expression and activity of the enzyme encoded by the gene corresponding to the KC-1 cDNA clone.

EXAMPLE 14-Transgenic plant phenotype analysis

pCGP1810 in Skr4 x SW63

The expression of the introduced KC-1 cDNA in the Skr4 x SW63 hybrid had a marked effect on flower colour. Ten of the twelve transgenic plants transformed with pCGP1810 produced flowers with an altered petal colour (RHSCC# 73A), compared with the Skr4 x Sw63 control (RHSCC# 74C). Moreover the anthers and pollen of the transgenic flowers were pink, compared with those of the control Skr4 x SW63 plant, which were white.. In addition, expression of the KC-1 cDNA in the Skr4 x SW63 hybrid conferred a dark pink hue to the corolla, which is normally pale lilac. The colour codes are taken from the Royal Horticultural Society's Colour Chart (RHSCC). They provide an alternative means by which to describe the colour phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colours and should not be regarded as limiting the possible colours which may be obtained.

Acid-hydrolysed floral extracts (see Example 11) were run in a Forestal solvent system (HOAc:water:HCl; 30: 10: 3) (Markham, 1982). The 3' hydroxylated flavonoids, peonidin and quercetin, were readily detected in the petal limbs of the transgenic plants. Only kaempferol and a small amount of malvidin were detected in the non-transgenic Skr4 x SW63 control.

The accumulation of the 3'-hydroxylated anthocyanidin, peonidin, in the petals of the transgenic Skr4 x SW63/pCGP1810 plants correlated with the dark pink colours observed in the petals of the same plants.

Construction of pCGP1813

Plasmid pCGP1811 was constructed by cloning the cDNA insert from pCGP1807 in a "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP1958. The plasmid pCGP1958 contains the Mac promoter and mannopine synthase (*mas*)(Comai *et al.*, 1990) terminator in a pUC19 backbone. The plasmid pCGP1807 was digested with PstI and XhoI to release the cDNA insert. The overhanging 5' ends were filled in using DNA

polymerase (Klenow fragment) (Sambrook *et al.*, 1989). The cDNA fragment was isolated and purified using the Bresaclean kit (Bresatec) and ligated with SmaI ends of the pCGP1958 vector to produce pCGP1811.

The plasmid pCGP1811 was subsequently digested with PstI to release the expression cassette containing the Mac promoter driving the KC-1 cDNA with a *mas* terminator, all of which were contained on a 4kb fragment. The expression cassette was isolated and ligated with PstI ends of the pWTT2132 binary vector (DNA Plant Technology Corporation; Oakland, California) to produce pCGP1813 (Figure 10).

Transformation of *Dianthus caryophyllus* cv. Kortina Chanel with the Carnation F3'H cDNA clone.

The binary vector pCGP1813 was introduced into *A. tumefaciens* strain AGL0 cells, as described in Example 9. The pCGP1813/AGL0 cells were used to transform carnation plants, to reduce the amount of 3'-hydroxylated flavonoids.

(a) Plant Material

Dianthus caryophyllus (cv. Kortina Chanel) cuttings were obtained from Van Wyk and Son Flower Supply, Victoria, Australia. The outer leaves were removed and the cuttings were sterilised briefly in 70% v/v ethanol followed by 1.25% w/v sodium hypochlorite (with Tween 20) for 6 min and rinsed three times with sterile water. All the visible leaves and axillary buds were removed under the dissecting microscope before co-cultivation.

(b) Co-cultivation of *Agrobacterium* and *Dianthus* Tissue

Agrobacterium tumefaciens strain AGL0 (Lazo *et al.*, 1991), containing the binary vector pCGP1813, was maintained at 4°C on LB agar plates with 50 mg/L tetracycline. A single colony was grown overnight in liquid LB broth containing 50 mg/L tetracycline and diluted to 5 x 10^8 cells/mL next day before inoculation. *Dianthus* stem tissue was co-cultivated with *Agrobacterium* for 5 days on MS medium supplemented with 3% w/v sucrose, 0.5 mg/L BAP, 0.5 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D), 100 mM acetosyringone

and 0.25% w/v Gelrite (pH 5.7).

(c) **Recovery of Transgenic *Dianthus* Plants**

For selection of transformed stem tissue, the top 6-8 mm of each co-cultivated stem was cut into 3-4 mm segments, which were then transferred to MS medium (Murashige and Skoog, 1962) supplemented with 0.3% w/v sucrose, 0.5 mg/L BAP, 0.5 mg/L 2,4-D, 1 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite. After 2 weeks, explants were transferred to fresh MS medium containing 3% sucrose, 0.16 mg/L thidiazuron (TDZ), 0.5 mg/L indole-3-butyric acid (IBA), 2 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.25% w/v Gelrite and care was taken at this stage to remove axillary shoots from stem explants. After 3 weeks, healthy adventitious shoots were transferred to hormone free MS medium containing 3% w/v sucrose, 5 μg/L chlorsulfuron, 500 mg/L ticarcillin, 0.25% w/v Gelrite. Shoots which survived 5 μg/L chlorsulfuron were transferred to the same medium for shoot elongation.

Elongated shoots were transferred to hormone-free MS medium containing 5 μg/L chlorsulfuron, 500 mg/L ticarcillin and 0.4% w/v Gelrite, in glass jars, for normalisation and root production. All cultures were maintained under a 16 hour photoperiod (120 mE/m^2/s cool white fluorescent light) at 23± 2°C. Normalised plantlets, approximately 1.5-2 cm tall, were transferred to soil (75% perlite/25% peat) for acclimation at 23°C under a 14 hour photoperiod (200 mE/m^2/s mercury halide light) for 3-4 weeks. Plants were fertilised with a carnation mix containing 1g/L $CaNO_3$ and 0.75 g/L of a mixture of microelements plus N:P:K in the ratio 4.7:3.5: 29.2.

EXAMPLE 15 -Isolation of a F3'H cDNA clone from *Antirrhinum majus* (Snapdragon) using a differential display approach

A novel approach was employed to isolate a cDNA sequence encoding F3'H from *Antirrhinum majus* (snapdragon). Modified methods based on the protocols for (i) isolation of plant cytochrome P450 sequences using redundant oligonucleotides (Holton *et al.* 1993)

and (ii) differential display of eukaryotic messenger RNA (Liang and Pardee, 1992) were combined, to compare the petal cytochrome P450 transcript populations between wild type (Eos$^+$) and F3'H mutant (eos$^-$)snapdragon lines. Direct cloning of differentially expressed cDNA fragments allowed their further characterisation by Northern, RFLP and sequence analysis to identify putative F3'H encoding sequences. A full-length cDNA was obtained using the RACE protocol of Frohman *et al.* (1988) and the clone was shown to encode a functional F3'H following both transient and stable expression in petunia petal cells.

Plant Material

The *Antirrhinum majus* lines used were derived from the parental lines K16 (eos$^-$) and N8 (Eos$^+$). K16 is a homozygous recessive mutant lacking F3'H activity, while N8 is wild type for F3'H activity. These lines are similar, though not isogenic. The seed of capsule E228^2 from the selfed K16 x N8 F_1 plant (#E228) was germinated and the resultant plants (K16 x N8 F_2 plants) were scored for the presence or absence of cyanidin, a product of F3'H activity (see Figures 1a and 1b). The presence of cyanidin could be scored visually, as the flowers were a crimson colour, unlike the mutant plants which were a pink colour (from pelargonidin-derived pigments). The accuracy of the visual scoring was confirmed by TLC analysis of the petal anthocyanins, carried out as described in Example 11.

Of 13 plants raised from the E228^2 seed, 9 (#3, #4, #5, #6, #7, #9, #10, #12, #15) produced flowers with cyanidin (Eos$^+$/Eos$^+$ and Eos$^+$/eos$^-$) while 4 (#8, #11, #13, #14) produced only pelargonidin-derived pigments (eos$^-$/eos$^-$).

Synthesis of cDNA

Total RNA was isolated from the leaves of plant #13 and petal tissue of plants #3, #5, and #12 of the *A. majus* K16 x N8 F_2 segregating population (E228^2) using the method of Turpen and Griffith (1986). Contaminating DNA was removed by treating 50 μg total RNA with 1 unit RQ1 RNase-free DNase (Promega) in the presence of 40 units RNasin® ribonuclease inhibitor (Promega) for 3 hours at 37°C in a buffer recommended by the manufacturers. The RNA was then further purified by extraction with phenol/chloroform/iso-

amyl alcohol (25:24:1) and subsequent ethanol precipitation.

Anchored poly(T) oligonucleotides, complementary to the upstream region of the polyadenylation sequence, were used to prime cDNA synthesis from *A. majus* petal and leaf RNA. The oligonucleotide sequences synthesized were (5'-3'):

polyT-anchA	TTTTTTTTTTTTTTTTA	SEQ ID NO:27
polyT-anchC	TTTTTTTTTTTTTTTTC	SEQ ID NO:28
polyT-anchG	TTTTTTTTTTTTTTTTG	SEQ ID NO:29

Two micrograms of total RNA and 100 pmol of the appropriate priming oligonucleotide were heated to 70°C for 10 minutes, then chilled on ice. The RNA/primer hybrids were then added to a reaction containing 20 units RNasin® (Promega), 25 nM each dNTP, 10 mM DTT and 1x Superscript buffer (BRL). This reaction was heated at 37°C for 2 minutes, then 200 units of Superscript™ reverse transcriptase (BRL) were added and the reaction allowed to proceed for 75 minutes, after which the reverse transcriptase was inactivated by heating the mixture at 95°C for 20 minutes.

Amplification of cytochrome P450 sequences using PCR

Cytochrome P450 sequences were amplified using redundant oligonucleotides (designed to be complementary to conserved regions near the 3' end of plant cytochrome P450 coding sequences) and polyT anchored oligonucleotides. A similar approach was previously used to generate cytochrome P450 sequences from *Petunia hybrida* and is described in US Patent Number 5,349,125.

Four oligonucleotides (referred to as upstream primers) were synthesized. These were derived from conserved amino acid regions in plant cytochrome P450 sequences. The oligonucleotides (written 5' to 3') were as follows:

WAIGRDP	TGG GCI ATI GGI (A/C)GI GA(T/C) CC

SEQ ID NO:30 SEQ ID NO:31

FRPERF AGG AAT T(T/C)(A/C) GIC CIG A(A/G)(A/C) GIT T

SEQ ID NO:32 SEQ ID NO:33

Pet Haem-New CCI TT(T/C) GGI GCI GGI (A/C)GI (A/C)GI ATI TG(T/G) (C/G)CI GG

SEQ ID NO:34

EFXPERF GAI TT(T/C) III CCI GAI (A/C)GI TT

SEQ ID NO:35 SEQ ID NO:36

The upstream primers were used with each of the polyT anchored oligonucleotides to generate cytochrome P450 sequences in polymerase chain reactions using cDNA as a template. Fifty pmol of each oligonucleotide was combined with 2 μM of each dNTP, 1.5 mM $MgCl_2$, 1x PCR buffer (Perkin Elmer), 5 μCi α-[^{33}P] dATP (Bresatec, 1500 Ci/mmol), 2.5 units AmpliTaq® DNA polymerase (Perkin Elmer) and 1/10th of the polyT-anchor primed cDNA reaction (from above). Reaction mixes (50 μL) were cycled 40 times between 94°C for 15 seconds, 42°C for 15 seconds, and 70°C for 45 seconds, following an initial 2 minute denaturation step at 94°C. Cycling reactions were performed using a Perkin Elmer 9600 Gene Amp Thermal Cycler.

DNA sequences were amplified using each upstream primer/anchored primer combination and the appropriately-primed cDNA template. Each primer combination was used with the cDNA from the petals of the E228^2 plants #3 and #5 (cyanidin-producing flowers) and #12 (non-cyanidin producing flowers). Reactions incorporating leaf cDNA from plant #13 (cyanidin-producing flowers) were also included, as negative controls, because F3'H activity is not present at a significant level in healthy, unstressed leaf tissues.

Differential display of cytochrome P450 sequences

^{33}P-labelled PCR fragments were visualised following separation on a 5% (w/v) polyacrylamide/urea denaturing gel (Sambrook *et al.* 1989). A ^{33}P-labelled M13mp18 sequencing ladder was included on the gel to serve as a size marker. The sequencing gel was dried onto Whatman 3MM paper and exposed to Kodak XAR film at room temperature.

Comparison of bands between cyanidin-producing petal samples and the non-cyanidin petal sample revealed 11 bands which represent mRNAs exclusively present in the cyanidin-producing petals. Of these 11 bands, only two were also present (at a reduced intensity) in the leaf sample.

Isolation and cloning of PCR fragments from sequencing gel

PCR products were purified from the dried sequencing gel and reamplified by the method described by Liang *et al.* (1993). Amplified cDNAs were purified, following electrophoretic separation on a 1.2% (w/v) agarose/TAE gel, using a Bresaclean kit (Bresatec). The purified fragments were then directly ligated into either commercially-prepared pCR-Script™vector (Stratagene) or EcoRV-linearised pBluescript® (Stratagene) which had been T-tailed using the protocol of Marchuk *et al.* (1990).

Sequence of F3'H PCR products

Each of the eleven cloned differential display PCR products (with inserts not exceeding 500 bp) was sequenced on both strands and compared to other known cytochrome P450 sequences involved in anthocyanin biosynthesis, using the FASTA algorithm of Pearson and Lipman (1988).

Of the eleven cDNAs cloned, two (Am1Gb and Am3Ga) displayed strong homology with the petunia OGR-38 F3'H sequence (Examples 4 to 11) and the F3'5'H sequences (Holton *et al.*, 1993). Conserved sequences between clones Am1Gb and Am3Ga suggested that they represented overlapping fragments of the same mRNA. Clone Am3Ga extends from the sequence encoding the haem-binding region of the molecule (as recognised by the "Pet Haem-New" oligonucleotide; SEQ ID NO:34) to the polyadenylation sequence. Clone

Am1Gb extends from the cytochrome P450 sequence encoding the conserved "WAIGRDP" amino acid motif (complementary to primer 1; SEQ ID NO:30 and SEQ ID NO:31) to an area in the 3' untranslated region which was spuriously recognised by the primer 1 ("WAIGRDP") oligonucleotide.

EXAMPLE 16- RFLP analysis of cytochrome P450 cDNAs

Restriction fragment length polymorphism (RFLP) analysis was again used to investigate linkage of the gene corresponding to cDNA clone Am3Ga to the presence, or absence, of cyanidin-producing activity in petals. A ^{32}P-labelled insert of Am3Ga was used to probe Southern blots of genomic DNA isolated from K16 x N8 F_2 segregating plants as well as the parental K16 and N8 lines. Analysis of EcoRV-digested genomic DNA from 13 plants of the K16 x N8 F_2 segregating population revealed hybridization only to the sequences of N8 and the K16 x N8 F_2 segregating lines which displayed floral cyanidin production (Figure 11). The K16 x N8 F_2 plants which produced only pelargonidin-derived pigments in their petals (including parental line, K16) showed no specific hybridization (Figure 11, lanes 2, 8, 11, 13, 14). These data indicate a possible deletion of the genomic sequences corresponding to Am3Ga in the mutant K16 plant and, therefore, at least a partial deletion of the F3'H gene in this line.

EXAMPLE 17- Northern analysis of cytochrome P450 cDNAs

Northern analysis was used to confirm the expression profiles of the putative cytochrome P450 fragments as shown by differential display. Ten micrograms of total petal RNA from eight of the K16 x N8 F_2 segregating population was separated on a 1.2% (w/v) agarose/formaldehyde gel (Sambrook *et al.* 1989) and transferred to HybondN nylon membrane (Amersham). Leaf RNA from the cyanidin-producing plant #13 was also included as a negative control in the Northern analysis. ^{32}P-labelled fragments of the cDNA insert from clone Am3Ga was used to probe the RNA blot.

The Am3Ga probe recognised an approximately 1.8 kb transcript which was only detectable in the petals of cyanidin-producing plants (plants #1, #3, #4, #5, #8). No transcript was detectable in the pelargonidin-producing petals (plants #6, #11, #12) or in the leaf sample from plant #13 (Figure 12).

These data, taken with those of the RFLP analysis, provide strong evidence that Am3Ga clone represents a cytochrome P450 gene which is responsible for F3'H activity in snapdragon. The total lack of a detectable transcript in the petals of non-cyanidin-producing lines supports the findings of the RFLP analysis, that the loss of cyanidin-producing activity in the K16 line (and the homozygous recessive plants of the K16 x N8 F_2 segregating population) is the result of a deletion in the F3'H structural gene.

EXAMPLE 18- Isolation of a full-length snapdragon F3'H cDNA

The Rapid Amplification of cDNA Ends (RACE) protocol of Frohman *et al.* (1988) was employed to isolate a full-length F3'H cDNA clone using sequence knowledge of the partial Am3Ga clone. A gene-specific primer ("SnapredRace A" -complementary to Am3Ga sequences 361 to 334) was synthesized to allow reverse transcription from petal RNA. A 3' amplification primer ("SnapredRace C" -complementary to Am3Ga (3'UTR) sequences 283 to 259) was also synthesized to bind just upstream of "SnapredRace A". A "poly(C)" primer was used to amplify sequences from the 5' end of the cDNA molecule.

The sequences of the oligonucleotides used were (written 5'-3'):

Snapred Race A CCA CAC GAG TAG TTT TGG CAT TTG ACC C
SEQ ID NO:37

Snapred Race C GTC TTG GAC ATC ACA CTT CAA TCT G
SEQ ID NO:38

PolyC race CCG AAT TCC CCC CCC CC
SEQ ID NO:39

"Snapred Race A-primed" petal cDNA was poly(G)-tailed and a 5' cDNA fragment amplified with primers "Snapred Race C" and "PolyC race" using the method of Frohman *et al.* (1988). Pfu DNA polymerase (0.15 unit) (Stratagene) was combined with 2.5 units AmpliTaq® DNA polymerase (Perkin Elmer) to increase the fidelity of the PCR reaction.

The resultant 1.71 kb DNA fragment (sdF3'H) was cloned directly into EcoRV-linearised pBluescript® (Stratagene) vector which had been T-tailed using the protocol of Marchuk *et al.* (1990). This plasmid was named pCGP246.

EXAMPLE 19- Complete sequence of snapdragon F3'H

Convenient restriction sites within the sdF3'H cDNA sequence of pCGP246 were exploited to generate a series of short overlapping subclones in the plasmid vector pUC19. The sequence of each of these subclones was compiled to provide the sequence of the entire sdF3'H RACE cDNA. The sdF3'H cDNA sequence was coupled with that from clone Am3Ga to provide the entire sequence of a snapdragon F3'H cDNA (SEQ ID NO:5). It contains an open reading frame of 1711 bases which encodes a putative polypeptide of 512 amino acids (SEQ ID NO:6).

The nucleotide and predicted amino acid sequences of the snapdragon sdF3'H clone were compared with: those of the petunia OGR-38 cDNA clone (SEQ ID NO:1 and SEQ ID NO:2); the petunia F3'5'H cDNA clones Hf1 and Hf2; and other petunia cytochrome P450 sequences isolated previously (US Patent Number 5,349,125). The sequence of sdF3'H was most similar to that of the petunia F3'H cDNA clone (OGR-38) representing the Ht1 locus from *P. hybrida*, having 69% similarity at the nucleic acid level, over 1573 nucleotides, and 72.2% similarity at the amino acid level, over 507 amino acids.

The Hf1 clone showed 57.3% similarity, over 1563 nucleotides and 49.3% similarity, over 491 amino acids, to the snapdragon sdF3'H clone, while the Hf2 clone showed 57.7% similarity, over 1488 nucleotides, and 50.8% similarity, over 508 amino acids, to the snapdragon sdF3'H clone.

The snapdragon sdF3'H sequence contains two "in frame" ATG codons which could act to initiate translation. Initiation from the first of these codons (position 91 of SEQ ID NO:5) gives a protein with an additional 10 N-terminal amino acids and would be favoured according to the scanning model for translation (Kozak, 1989).

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 20- Transient expression of sdF3'H in plants

Construction of pCGP250

Plasmid pCGP250 (Figure 13) was created by cloning the entire sdF3'H RACE cDNA insert (from position 1 to 1711 (SEQ ID NO:5)) from pCGP246 in the "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP293 (Brugliera *et al.*, 1994). The plasmid pCGP246 was digested with EcoRI to release the cDNA insert. The cDNA fragment was blunt-ended by repairing the overhangs with the Klenow fragment of DNA polymerase I (Sambrook *et al.*, 1989) and purified, following agarose gel electrophoresis, using a Bresaclean kit (Bresatec). The blunt cDNA fragment was then ligated into the binary vector pCGP293, which had been linearised with XbaI and blunt-ended using the Klenow fragment of DNA polymerase I. The ligation was carried out using the Amersham ligation kit. Correct insertion of the insert in pCGP250 was established by BamHI and PstI restriction enzyme analysis of DNA isolated from gentamycin-resistant transformants.

Construction of pCGP231

Plasmid pCGP231 (Figure 14) was created by cloning the RACE cDNA insert from pCGP246, downstream of the first "in-frame" ATG codon (from position 104 to 1711 (SEQ ID NO:5), in the "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP293 (Brugliera *et al.*, 1994). The plasmid pCGP246 was digested with SspI (which recognises a site between the candidate ATG codons) and SmaI (with a site in the vector polylinker sequence) to release a blunt-ended cDNA fragment which includes the entire coding region downstream from the second putative initiation codon. The cDNA fragment was then ligated into the binary vector pCGP293, which had been linearised with XbaI and blunt-ended using the Klenow fragment of DNA polymerase I. The ligation was carried out using the Amersham ligation kit. Correct insertion of the insert in pCGP231 was established by BamHI and PstI restriction enzyme analysis of DNA isolated from gentamycin-resistant transformants.

Transient Expression Studies

To determine rapidly whether the pCGP246 sequences in pCGP231 and pCGP250 encoded active flavonoid 3'-hydroxylases in plants, a transient expression study was undertaken. Petals of the mutant *P. hybrida* line Skr4 X SW63 were bombarded with gold particles (1μm diameter) coated with either pCGP231 or pCGP250 plasmid DNA, using the method described in Example 8.

After 6-12 hours under lights in a controlled plant growth room at 22°C, red anthocyanin spots were observed on the surface of the petal tissue bombarded with pCGP231 coated particles. No coloured spots were observed in petals bombarded with pCGP250 or control petals bombarded with gold particles alone. These results indicated that the pCGP246 coding region (starting at the second ATG, position 121 of SEQ ID NO:5), under the control of the Mac promoter, was functional in petal tissue.

EXAMPLE 21- Stable expression of the snapdragon F3'H cDNA clone in petunia petals- Complementation of a ht1/ht1 petunia cultivar

The binary vectors pCGP250 and pCGP231 were introduced into *A. tumefaciens* strain AGL0 cells, as described in Example 9. The pCGP250/AGL0 and pCGP231/AGL0 cells were used to transform Skr4 x SW63 petunia plants (also described in Example 9), to test for stable expression and activity of the enzyme encoded by the gene corresponding to the snapdragon sdF3'H cDNA clone.

Three of the nine transgenic plants transformed with pCGP250 produced flowers with a slightly-altered petal colour (RHSCC# 73A), compared with the Skr4 x Sw63 control (RHSCC# 75C). Of the 11 transgenic plants transformed with pCGP231, one plant produced flowers with an altered petal colour (RHSCC# 73B). The anthers and pollen of the transgenic flowers were also white, as in the control. The codes are taken from the Royal Horticultural Society's Colour Chart (RHSCC). They provide an alternative means by which to describe the colour phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colours and should not be regarded as limiting the possible colours which may be obtained.

TLC analysis of floral extracts

Acid-hydrolysed floral extracts (see Example 11) were run in a Forestal solvent system (HOAc:water:HCl; 30: 10: 3) (Markham, 1982). Introduction of the sdF3'H cDNA clone into Skr4 x SW63 led to the production of increased levels of the 3'-hydroxylated flavonoid, peonidin, in the petals. Peonidin is the methylated derivative of cyanidin (Figures 1a and 1b).

EXAMPLE 22- Isolation of a F3'H cDNA clone from *Arabidopsis thaliana* using a PCR approach

In order to isolate a cDNA clone representing flavonoid 3'-hydroxylase from *Arabidopsis thaliana*, PCR fragments were generated using primers from the conserved regions of cytochrome P450s. One PCR product (p58092.13) was found to have high sequence similarity with the petunia OGR-38 and snapdragon F3'H cDNA clones. The PCR fragment

was then used, together with the Ht1 cDNA insert (OGR-38) from pCGP1805, to screen an *A. thaliana* cDNA library.

Design of oligonucleotides

Degenerate oligonucleotides for PCR DNA amplification were designed from the consensus amino acid sequence of *Petunia hybrida* cytochrome P450 partial sequences situated near the haem-binding domain. Primer degeneracy was established by the inclusion of deoxyinosine (designated as I below) in the third base of each codon (deoxyinosine base pairs with similar efficiency to A, T, G, and C), and the inclusion of alternate bases where the consensus sequences were non-specific. Thus, the amino-terminal directional primer "Pet Haem" (Petunia haem-binding domain), containing the cysteine residue codon crucial for haem binding, and the upstream primer "WAIGRDP" (See also Example 15) were designed.

WAIGRDP TGG GCI ATI GGI (A/C)GI GA(T/C) CC
SEQ ID NO:30 SEQ ID NO:31

Pet Haem CCI GG(A/G) CAI ATI C(G/T)(C/T) (C/T)TI CCI GCI CC(A/G) AAI GG
SEQ ID NO:40

Generation of cytochrome P450 sequences using PCR

Genomic DNA was isolated from *A. thaliana* ecotype Columbia, using the method described by Dellaporta *et al.* (1987). Polymerase chain reactions for amplification of cytochrome P450 homologues typically contained 100-200 ng of Columbia genomic DNA, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM each dNTP, 312 ng "WAIGRDP" and 484 ng "Pet Haem" and 1.25 units Taq polymerase (Cetus). Reaction mixes (50 μL) were cycled 40 times between 95°C for 50 seconds, 45°C for 50 seconds and 72°C for 45 seconds.

The expected size of specific PCR amplification products, using the "WAIGRDP" and "Pet Haem" primers on a typical P450 gene template, without an intron, is approximately 150

base pairs. PCR fragments of approximately 140 to 155 base pairs were isolated and purified using the Mermaid® kit (BIO 101). The PCR fragments were re-amplified to obtain enough product for cloning and then end-repaired using Pfu DNA polymerase and finally cloned into pCR-Script™Direct SK(+) (Stratagene). The ligated DNA was then used to transform competent DH5α cells (Inoue *et al.*, 1990).

Sequence of PCR products

Plasmid DNA from 15 transformants was prepared (Del Sal *et al.*, 1989). Sequencing data generated from these PCR fragments indicated that 11 out of the 15 represented unique clones. A distinct set of cytochrome P450 consensus amino acids was also found in the translated sequence encoded within the *A. thaliana* PCR inserts. The sequences of the PCR fragments were also compared with those of the petunia OGR-38 F3'H cDNA clone and the snapdragon F3'H cDNA clone. The PCR fragment, p58092.13, was most similar to the F3'H sequences from both petunia and snapdragon.

EXAMPLE 23- Screening of *A. thaliana* cDNA library

To isolate a cDNA clone of the p58092.13 PCR product, an *A. thaliana* ecotype Columbia cDNA library (Newman *et al.*, 1994; D' Alessio *et al.*, 1992) was screened with a ^{32}P-labelled fragment of p58092.13 together with a ^{32}P-labelled fragment of the petunia Ht1 cDNA insert (OGR-38), contained in pCGP1805.

A total of 600,000 pfu was plated at a density of 50,000 pfus per 15 cm diameter plate, as described by D' Alessio *et al* (1992). After phage growth at 37°C plates were stored at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen™filters (DuPont) and treated as recommended by the manufacturer.

Prior to hybridization, the duplicate plaque lifts were washed in prewashing solution (50 mM Tris-HCl pH7.5, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) sarcosine) at 65°C for 30 minutes; stripped in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of

0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% (w/v) SDS.

Hybridization conditions included a prehybridization step in 50% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragment of p58092.13 ($2x10^6$cpm/mL) was then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% (w/v) SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Eleven strongly-hybridizing plaques were picked into PSB and rescreened as detailed above, to isolate purified plaques. These filters were also probed with ^{32}P-labelled fragment of the petunia Ht1 cDNA insert (OGR-38), contained in pCGP1805, under low stringency conditions. Low stringency conditions included prehybridization and hybridization at 42°C in 20% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS and washing in 6xSSC, 1% (w/v) SDS (w/v) at 65°C for 1 hour.

The OGR-38 and p58092.13 probes hybridized with identical plaques. The 11 pure plaques were picked into PSB and the plasmid vectors pZL1 containing the cDNA clones were rescued using the bacterial strain DH10B(Zip). Plasmid DNA was prepared (Del Sal *et al.*, 1989) and the cDNA inserts were released upon digestion with BamHI and EcoRI. The 11 plasmids contained cDNA inserts of between 800bp and 1 kb. Sequence data generated from the 5' region of the cDNA inserts suggested that nine of these clones were identical. Sequence data were generated from the 5' ends of all nine cDNA inserts and the 3' end of only one cDNA insert. The sequence data generated from all clones were compiled to produce the nucleotide and translated sequence shown as SEQ ID NO:7 and SEQ ID NO:8.

The *A. thaliana* putative F3'H sequences were compared with the sequences of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2) and was 64.7% similar to the petunia F3'H cDNA clone, over 745 nucleotides, and 63.7 % similar, over 248 amino

acids.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

Isolation of a F3'H genomic clone from *Arabidopsis thaliana*

To isolate a genomic clone of the *A. thaliana* F3'H gene, a *A. thaliana* ecotype Landsberg erecta genomic DNA library was screened with ^{32}P-labelled p60606.04 fragments. The library was created by cloning partial MboI-digested genomic DNA between BamHI-digested bacteriophage lambda EMBL4 arms. The primary library, which contained 30,000 clones, was amplified once before screening.

The p60606.04 clone, containing a 1 kb fragment of *A. thaliana* F3'H cDNA, was digested with BamHI/EcoRI to excise the insert which was purified using GeneClean (Bio 101). Probe was ^{32}P-labelled using the nick-translation procedure (Sambrook et al., 1989). Approximately 20,000 plaques were probed at high stringency (50% formamide at 37^{o} C) and filters were washed in: 2x SSPE; 2x SSPE, 0.1% (w/v) SDS; 0.1x SSPE, all at 65°C. Re-screening was carried out under the same conditions.

DNA was purified from three positive plaques (λTT7-1, λTT7-5 and λTT7-6) and mapped by digestion with EcoRI and EcoRI/SalI. All three clones had an EcoRI fragment in common. λTT7-1 and λTT7-5 had overlapping but not identical restriction patterns. A Southern blot of these digests was probed as above and, for λTT7-1 and λTT7-5, a common 6.5 kb EcoRI/SalI fragment hybridized. A smaller EcoRI/SalI fragment in λTT7-6 also hybridized and was presumably at the insert boundary.

EcoRI/SalI fragments from lTT7-5 were cloned into pBlueScript SK+ and a clone containing

the 6.5 kb fragment, designated E-5, was identified by hybridization (as above) and insert size. A restriction map was compiled for the fragment using EcoRI, SalI, KpnI, HindIII and BglII in various combinations, and by hybridization to Southern blots of these digests with the BamHI/EcoRI insert from the *A. thaliana* F3'H cDNA clone.

Complete sequence of Tt7 genomic clone

A 6.4 kb BamHI fragment from pTt7-2, containing most of the Tt7 genomic fragment was purified, self-ligated, sonicated, end-repaired, size-fractionated (450bp to 800bp) and cloned into SmaI-cut pUC19 using standard techniques (Sambrook et al., 1989). Recombinant clones were isolated, and plasmid DNA was purified and sequenced using M13-21 or M13 reverse sequencing primers. The sequence from overlapping clones was combined into one contiguous fragment. The sequence of the ends of the Tt7 genomic fragment were also obtained by sequencing with the -21 and REV primers. All of the sequences were combined together to obtain the complete sequence of the 6.5 kb EcoRI/SalI fragment from E-5 (SEQ ID NO:9).

The sequences over the coding region of the arabidopsis Tt7 genomic clone (SEQ ID NO:10, 11, 12 and 13) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and 2). The arabidopsis Tt7 coding region showed 65.4% similarity, over 1066 nucleotides, and 67.1% similarity, over 511 amino acids, to that of the petunia OGR-38 F3'H cDNA clone.

Transformation of a tt7 Arabidopsis mutant

Preparation of binary vector

The EcoRI/SalI fragment from E-5 was cloned into EcoRI/SalI-cut pBI101 (Jefferson *et al.*, 1987). Two separate but identical clones were identified: pBI-Tt7-2 (Figure 15) and pBI-Tt7-4. Both clones were used for transformation of *A. tumefaciens*.

Plant Transformation

Plasmids pBI-Tt7-2, pBI-Tt7-4 and pBI101 were transformed into *Agrobacterium* strain

GV3101 pMP90 by electroporation. Transformants were selected on medium containing 50 μg/mL kanamycin (and 50 μg/mL gentamycin to select for the resident pMP90).

Plasmid DNA, from four transformant colonies for each clone, was isolated and digested with EcoRI/SalI, electrophoresed, Southern blotted, and probed with the Tt7 cDNA insert. For pBI-Tt7-2 and pBI-Tt7-4, the expected insert band was identified.

One transformant for each plasmid (i.e.: one control [pBI101 C4], one each of the two Tt7 clones [pBI-Tt7-2-3 and pBI Tt7-4-4]) was used to vacuum infiltrate the *A. thaliana* tt7 mutant line NW88 (4 pots of 10 plants each for each construct), using the a method essentially as described by Bechtold *et al.* (1993).

Seed from each pot was harvested. One hundred mg of seed (approximately 5,000) was plated on nutrient medium (described by Haughn and Somerville, 1986) containing 50 μg/mL kanamycin. Kanamycin-resistant transformants were visible after 7 to 10 days. In the case of pBI-Tt7-2-3 and pBI-Tt7-4-4, a total of 11 transformants were isolated from 5 different seed lots (i.e.: pots) and all kanamycin-resistant transformants were visibly Tt7 in phenotype and exhibited the characteristic red/purple anthocyanin pigments at the margins of the cotyledons and at the hypocotyl. A single kanamycin-resistant transformant was isolated from only one of the four pots of control transformants and it did not exhibit a "wild-type" Tt7 phenotype.

Complementation of tt7 mutant

These transformants were planted out and grown to maturity and individually harvested for seed. In each case, for pBI-Tt7-2-3 and pBI-Tt7-4-4 transformants, the seeds were visibly more brown than the pale brown seed of the tt7 mutant plants. The seed from the control transformant was indistinguishable from the tt7 mutant parent. These seed were plated out on nutrient medium and nutrient medium with kanamycin added, and scored for the Tt7 phenotype (red/purple anthocyanin pigments at the margins of the cotyledons and at the hypocotyl) and kanamycin resistance. The progeny of at least one transformant for each seed

lot was examined, since these were clearly independent transformation events.

Without exception, kanamycin-resistant seedlings exhibited the Tt7 phenotype while kanamycin-sensitive individuals were tt7. In some cases, kanamycin resistance was weak and variable among a family of seed and it was difficult to unequivocally determine whether individuals were kanamycin resistant or kanamycin sensitive.

EXAMPLE 24- Isolation of a F3'H cDNA clone from *Rosa hybrida*

In order to isolate a Rose F3'H cDNA clone, a *Rosa hybrida* cv. Kardinal petal cDNA library was screened with ^{32}P-labelled fragments of the petunia Ht1 cDNA clone (OGR-38), contained in pCGP1805, and snapdragon F3'H cDNA clone (sdF3'H), contained in pCGP246.

Construction of a petal cDNA library from Rose cv. Kardinal

Total RNA was prepared from the buds of *Rosa hybrida* cv. Kardinal stage 2. At this stage, the tightly closed buds were 1.5 cm high and approximately 0.9 cm wide with pale pink petals.

Frozen tissue (1-3 g) was ground in liquid nitrogen with a mortar and pestle, placed in 25 mL pre-chilled Buffer A [0.2 M boric acid, 10 mM EDTA (sodium salt) (pH 7.6)] and homogenized briefly. The extract was mixed on a rotary shaker until it reached room temperature and an equal volume of phenol/chloroform (1:1 v/v), equilibrated with Buffer A, was added. After mixing for a further 10 minutes, the RNA preparation was centrifuged at 10,000 x *g* for 10 minutes at 20°C. The upper aqueous phase was retained and the phenol interface re-extracted as above. The aqueous phases were pooled and adjusted to 0.1 M sodium acetate (pH 6.0), 2.5 volumes 95% ethanol were added and the mixture was stored at -20°C overnight.

The preparation was centrifuged at 10,000 x *g* for 10 minutes at 4°C, the pellet dissolved

gently in 20 mL Buffer B [25 mM boric acid, 1.25 mM EDTA (sodium salt), 0.1 M NaCl (pH 7.6)] and 0.4 volumes 2-butoxyethanol (2BE) were added. This solution was incubated on ice for 30 minutes. It was then centrifuged at 10,000 x *g* for 10 minutes at 0°C and the supernatant was carefully collected. After addition of 1.0 volume of 2BE and incubation on ice for a further 30 minutes, the supernatant was again centrifuged at 10,000 x *g* for 10 minutes at 0°C. The resulting pellet was gently washed with Buffer A:2BE (1:1 v/v), then with 70% (v/v) ethanol, 0.1 M potassium acetate and finally with 95% ethanol. The pellet was air dried and dissolved in 1 mL diethyl pyrocarbonate (DEPC)-treated water. This was adjusted to 3 M lithium chloride, left on ice for 60 minutes and centrifuged at 10,000 x *g* for 10 minutes at 0°C. The pellet was washed twice with 3 M LiCl and then with 70% ethanol, 0.1 M potassium acetate.

The resulting RNA pellet was dissolved in 400 μL DEPC-treated water and extracted with an equal volume phenol/chloroform. The RNA mix was then centrifuged at 10,000 x *g* for 5 minutes at 20°C, the aqueous phase collected and made to 0.1 M sodium acetate, and a further 2.5 volumes of 95% ethanol were added. After 30 minutes incubation on ice, the mix was centrifuged at 13,000 rpm (5,000 x *g*) for 20 minutes at 20°C and the RNA pellet resuspended gently in 400 μL DEPC-treated water.

Poly (A)$^+$ RNA was selected from the total RNA by Oligotex dT-30 (Takara, Japan) following the manufacturer's protocol. The cDNA was synthesized according to the method in Brugliera *et al.* (1994) and used to construct a non-directional petal cDNA library in the EcoRI site of λZAPII (Stratagene). The total number of recombinants obtained was 3.5 x 10^5.

After transfecting XL1-Blue cells, the packaged cDNA mixture was plated at 50,000 pfu per 15 cm diameter plate. The plates were incubated at 37°C for 8 hours, and the phage were eluted in 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 8.0, 0.01% (w/v) gelatin (Phage Storage Buffer (PSB)) (Sambrook *et al.*, 1989). Chloroform was added and the phage stored at 4°C as an amplified library.

200,000 pfus of the amplified library were plated onto NZY plates (Sambrook *et al.*, 1989) at a density of 10,000 pfu per 15 cm plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After incubation at 4°C overnight, duplicate lifts (labelled as group A and group B) were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Screening of Kardinal cDNA library for a F3'H cDNA clone

Prior to hybridization, the duplicate plaque lifts were washed in prewashing solution (50 mM Tris-HCl pH7.5, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) sarcosine) at 65°C for 30 minutes; stripped in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% (w/v) SDS.

The group A filters of the duplicate lifts from the Kardinal cDNA library were screened with ^{32}P-labelled fragments of an NcoI fragment from pCGP1805 containing the petunia Ht1 (OGR-38) cDNA clone, while the group B filters were screened with ^{32}P-labelled fragments of EcoRI/SspI fragment from pCGP246 containing the snapdragon F3'H clone.

Hybridization conditions included a prehybridization step in 10% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragment ($2x10^6$cpm/mL) was then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed at 42°C in 2 x SSC, 1% (w/v) SDS for 2 hours followed by 1 x SSC, 1% (w/v) SDS for 1 hour and finally in 0.2 x SSC/1% (w/v) SDS for 2 hours. The filters were exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Four strongly-hybridizing plaques (R1, R2, R3, R4) were picked into PSB and rescreened to isolate pure plaques. The plasmids contained in the λZAP bacteriophage vector were rescued and digested with EcoRI to release the cDNA inserts. Clone R1 contained a 1.0 kb insert while clones R2, R3 and R4 contained inserts of approximately 1.3 kb each. Sequence

data were generated from the 3' and 5' ends of the R4 cDNA insert.

The rose R4 putative F3'H sequence was compared with that of the petunia OGR-38 F3'H sequence. At the nucleotide level, the R4 cDNA clone showed 63.2% and 62.1% similarity over 389 nucleotides at the 5' end and 330 nucleotides at the 3' end, respectively. At the amino acid level, the R4 clone showed 65.4 % and 73.9% similarity over 130 amino acids at the 5' end and 69 amino acids at the 3' end, respectively. Based on the high sequence similarity of the Rose R4 cDNA clone to that of the petunia F3'H cDNA clone (OGR-38), a corresponding "full-length" cDNA clone was isolated, as described in Example 25, below.

EXAMPLE 25- Isolation of a full-length rose F3'H cDNA

In order to isolate a "full-length" F3'H cDNA clone from Rose, the *Rosa hybrida* cv Kardinal petal cDNA library described in Example 24 was screened with ^{32}P-labelled fragments of the rose R4 cDNA clone, described above.

A total of 1.9 x 10^6 pfus of the amplified library were plated onto NZY plates at a density of 100,000 pfus per 15 cm diameter plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After incubation at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Screening of Kardinal cDNA library for full-length F3'H cDNA clones

Prior to hybridization, the duplicate plaque lifts were treated as described in Example 24.

The duplicate lifts from the Kardinal cDNA library were screened with ^{32}P-labelled fragments of an EcoRI fragment from the rose R4 cDNA clone.

Hybridization conditions included a prehybridization step in 50% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulphate, 1% (w/v) SDS at 42°C for at least 1 hour. The ^{32}P-labelled fragment of the rose R4 cDNA clone (1x10^6cpm/mL) was then added to the

hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% (w/v) SDS at 42°C for 2 x 1 hour and exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

Seventy-three strongly-hybridizing plaques (1-73) were picked into 1mL of PSB and stored at 4°C overnight. 100μL of each was then aliquoted into a microtitre tray as an ordered array.

XL1-Blue MRF' cells were added to 10mL of molten NZY top agar, poured onto NZY plates (15cm diameter) and allowed to set. A replica plating device was used to transfer the 73 phage isolates in an ordered array onto the NZY plate previously inoculated with the XL1-Blue MRF' cells. After incubation at 37°C for 6 hours followed by 4°C overnight, triplicate lifts (arrays 1, 2 and 3) were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Prior to hybridization, the duplicate plaque lifts were treated as described in Example 24.

The 3 arrays were screened with ^{32}P-labelled fragments of a) an EcoRI/SalI fragment covering the 5' end of the rose R4 cDNA clone, b) an EcoRI/ClaI fragment covering the 5' end of the rose R4 cDNA clone or c) an EcoRI fragment of the entire rose R4 cDNA clone using the hybridisation and washing conditions described above, except that the final wash was in 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 30 minutes. The filters were exposed to Kodak XAR film with an intensifying screen at -70°C for 16 hours.

All 73 plaques hybridised with the full R4 cDNA clone (EcoRI fragment) whilst a total of only 17 hybridised with the 5' end of the R4 cDNA clone (either EcoRI/SalI or the EcoRI/ClaI fragments). The 17 phage isolates were rescreened as described above to isolate purified plaques. Pure plaques were obtained from 9 out of the 17 (2, 4, 26, 27, 34, 38, 43, 44, 56). The plasmids contained in the λZAP bacteriophage vector were rescued and the sizes of the cDNA inserts were determined using an EcoRI digestion. The cDNA inserts

ranged from 0.9kb to 1.9kb. Of the nine, only #34 (named pCGP2158) and #38 (named pCGP2159) contained cDNA inserts of approximately 1.9kb. Sequence data were generated from the 3' and 5' ends of the cDNA inserts and showed that clones #34 and #38 represented the same gene.

The complete sequence of the rose cDNA clone (#34) contained in the plasmid pCGP2158 was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989). The sequence (SEQ ID NO:14) contained an open reading frame of 1696 bases which encodes a putative polypeptide of 520 amino acids (SEQ ID NO:15).

The nucleotide and predicted amino acid sequences of the rose F3'H #34 cDNA clone (SEQ ID NO:14 and SEQ ID NO:15) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2) and the snapdragon sdF3'H clone (SEQ ID NO:3 and SEQ ID NO:4). The rose F3'H #34 cDNA clone showed 64.7% similarity ,over 1651 nucleotides, and 72.7% similarity, over 509 amino acids, to that of the petunia OGR-38 cDNA clone, and 67.2% similarity, over 1507 nucleotides, and 68.9 similarity, over 502 amino acids, to that of the snapdragon sdF3'H clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 26- Stable expression of the rose F3'H cDNA clone (#34) in petunia petals- Complementation of a ht1/ht1 petunia cultivar

Preparation of pCGP2166

Plasmid pCGP2166 (Figure 16) was constructed by cloning the cDNA insert from pCGP2158 in a "sense" orientation behind the Mac promoter (Comai *et al.*, 1990) of pCGP293 (Brugliera *et al.*, 1994). The plasmid pCGP2158 was digested with EcoRI to release the cDNA insert. The overhanging 5' ends were filled in using DNA polymerase (Klenow fragment) (Sambrook *et al.*, 1989). The cDNA fragment was isolated and ligated with filled in BamHI ends of the pCGP293 binary vector. Correct insertion of the fragment in pCGP2166 was established by restriction enzyme analysis of DNA isolated from gentamycin-resistant transformants.

The binary vector pCGP2166 was introduced into *A. tumefaciens* strain AGL0 cells, as described in Example 9. The pCGP2166/AGL0 cells were then used to transform Skr4 x SW63 petunia plants (also described in Example 9), to test for stable expression and activity of the enzyme encoded by the gene corresponding to the rose #34 cDNA clone.

EXAMPLE 27- Transgenic plant phenotype analysis

pCGP2166 in Skr4 x SW63

The expression of the introduced rose F3'H cDNA in the Skr4 x SW63 hybrid had a marked effect on flower colour. The stamen tissue of the non-transgenic control is white, whereas the same tissue in most of the transgenic plants was pink. In addition, expression of the rose F3'H cDNA in the Skr4 x SW63 hybrid conferred a dark pink hue (RHSCC# 64C and 74C) to the corolla, which is normally pale lilac (RHSCC# 75C). The colour codes are taken from the Royal Horticultural Society's Colour Chart (RHSCC). They provide an alternative means by which to describe the colour phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colours and should not be regarded as limiting the possible colours which may be obtained.

Acid-hydrolysed floral extracts (see Example 11) were run in a Forestal solvent system (HOAc:water:HCl; 30: 10: 3) (Markham, 1982). The 3' hydroxylated flavonoids, peonidin and quercetin, were readily detected in the petal limbs of the transgenic plants. Only kaempferol and a small amount of malvidin were detected in the non-transgenic Skr4 x SW63

control.

The accumulation of the 3'-hydroxylated anthocyanidin, peonidin and the flavonol, quercetin, in the petals of the transgenic Skr4 x SW63/pCGP2166 plants correlated with the pink and dark pink colours observed in the petals of the same plants.

Preparation of pCGP2169

The binary construct pCGP2169 (Figure 17) was prepared by cloning the cDNA insert from pCGP2158 in a "sense" orientation between the CaMV35S promoter (Franck *et al.*, 1980; Guilley *et al.*, 1982) and *ocs* terminator (De Greve *et al.*, 1982). The plasmid pCGP1634 contained a CaMV35S promoter, β-glucuronidase (GUS) reporter gene encoded by the *E. coli uid*A locus (Jefferson *et al.*, 1987) and *ocs* terminator region in a pUC19 vector. The plasmid pCGP2158 was digested with NcoI/XbaI to release the cDNA insert. The plasmid pCGP1634 was also digested with NcoI/XbaI to release the backbone vector containing the CaMV35S promoter and the *ocs* terminator. The fragments were isolated and ligated together to produce pCGP2167. The plasmid pCGP2167 was subsequently digested with PvuII to release the expression cassette containing the CaMV35S promoter, the rose F3'H cDNA clone and the *ocs* termintor. This expression cassette fragment was isolated and ligated with SmaI ends of pWTT2132 binary vector (DNA Plant Technology Corporation; Oakland, California) to produce pCGP2169 (Figure 17).

The binary vector pCGP2169 was introduced into *A. tumefaciens* strain AGL0 cells, as described in Example 9. The pCGP2169/AGL0 cells are used to transform rose plants, to reduce the amount of 3'-hydroxylated flavonoids.

EXAMPLE 28- Isolation of a putative F3'H cDNA clone from chrysanthemum

In order to isolate a chrysanthemum F3'H cDNA clone, a chrysanthemum cv. Red Minstral petal cDNA library was screened with ^{32}P-labelled fragments of the petunia Ht1 cDNA clone (OGR-38), contained in pCGP1805.

Construction of a petal cDNA library from chrysanthemum cv. Red Minstral

Total RNA was prepared from the petals (stages 3 to 5) of chrysanthemum cv. Red Minstral using Trizol™reagent (Life Technologies) (Chomczynski and Sacchi, 1987) according to the manufacturer's recommendations. Poly(A)+ RNA was enriched from the total RNA, using a mRNA isolation kit (Pharmacia) which relies on oligo-(dT) affinity spun-column chromatography .

A Superscript™cDNA synthesis kit (Life Technologies) was used to construct a petal cDNA library in ZipLox using 5 μg of poly(A)+ RNA isolated from stages 3 to 5 of Red Minstral as template.

30,000 pfus of the library were plated onto LB plates (Sambrook *et al.*, 1989) at a density of 3,000 pfus per 15 cm plate after transfecting Y1090r-, and incubated at 37°C for 16 hours. After incubation at 4°C for one hour, duplicate lifts were taken onto Hybond N+™filters (Amersham) and treated as recommended by the manufacturer.

Screening of the Red Minstral cDNA Library

The duplicate lifts from the Red Minstral petal cDNA library were screened with ^{32}P-labelled fragments of the 1.8 kb Asp718/BamHI insert from pCGP1805.

Hybridization conditions included a prehybridization step in 1mM EDTA (pH8.0), 0.5MNa_2HPO_4 (pH7.2), 7% (w/v) SDS (Church and Gilbert, 1984) at 65°C for at least 1 hour. The ^{32}P-labelled fragments ($1x10^6$cpm/mL) were then added to the hybridization solution and hybridization was continued at 65°C for a further 16 hours. The filters were then washed in 2 x SSC, 0.1% (w/v) SDS at 65°C for 2 x 1 hour and exposed to Kodak BioMax™film with an intensifying screen at -70°C for 48 hours.

Eight strongly-hybridizing plaques were picked into PSB (Sambrook *et al.*, 1989). Of these, 2 (RM6i and RM6ii) were rescreened to isolate purified plaques, using the hybridization conditions as described for the initial screening of the cDNA library. The plasmids

contained in the λZipLox bacteriophage vector were rescued according to the manufacturer's protocol and sequence data was generated from the 3' and 5' ends of the cDNA inserts. The partial sequences of the RM6i and RM6ii cDNA inserts were compared with the complete sequence of the petunia OGR-38 F3'H cDNA clone. The RM6i cDNA clone showed relatively high sequence similarity with that of the petunia OGR-38 cDNA clone, and was further characterised.

The RM6i cDNA insert contained in pCHRM1 was released upon digestion with EcoRI and was approximately 1.68 kb. The complete sequence of RM6i cDNA clone (SEQ ID NO:16) contained in the plasmid pCHRM1 was determined by compilation of sequence from subclones of the RM6i cDNA insert.

The nucleotide and predicted amino acid sequences of the chrysanthemum RM6i cDNA insert (SEQ ID NO:16 and SEQ ID NO:17) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2). The sequence of the chrysanthemum RM6i cDNA insert showed 68.5% similarity, over 1532 nucleotides, and 73.6 % similarity, over 511 amino acids, to that of the petunia OGR-38 F3'H cDNA clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

Construction of pLN85 (antisense binary)

A plasmid designated pLN84 was constructed by cloning the RM6i cDNA insert from pCHRM1 in the "antisense" orientation behind the complete CaMV35S promoter contained in pART7 (Gleave 1992). The plasmid pCHRM1 was digested with NotI to release the cDNA insert. The RM6i cDNA fragment was blunt-ended using T4 DNA polymerase (Sambrook et al., 1989) and purified, following agarose gel electrophoresis and GELase

(Epicentre Technologies). The purified fragment was ligated with SmaI ends of the pART7 shuttle vector to produce pLN84. The plasmid pLN84 was subsequently digested with NotI to release the expression cassette containing CaMV35S: RM6i cDNA: ocs. The expression cassette was isolated as a single fragment and ligated with NotI ends of the pART27 binary vector (Gleave, 1992) to produce pLN85 (Figure 18). Correct insertion of the fragment was established by restriction enzyme analysis of DNA isolated from streptomycin-resistant *E.coli* transformants.

The binary vector pLN85 is introduced into chrysanthemum plants via *Agrobacterium*-mediated transformation, as described in Ledger *et al,* 1991), to reduce the amount of 3'-hydroxylated flavonoids.

EXAMPLE 29- Isolation of a putative F3'H cDNA clone from *Torenia fournieri*

In order to isolate a torenia F3'H cDNA clone, the petunia Ht1-linked F3'H cDNA clone (OGR-38), contained in pCGP1805, was used to screen a *Torenia fournieri* cv. Summer Wave petal cDNA library, under low stringency conditions.

Construction of *Torenia fournieri* cv. Summer Wave petal cDNA library

A directional petal cDNA library was prepared from Summer Wave flowers, essentially as described in Example 4.

Screening of Summer Wave petal cDNA library

Lifts of a total of 200,000 of the amplified Summer Wave petal cDNA library were screened with DIG-labelled fragments of the 1.8 kb OGR-38 cDNA insert from pCGP1805. A DIG DNA labelling and detection kit from Boehringer-Mannheim was used according to the manufacturer's recommendations.

Hybridizations were carried out in 30% (v/v) formamide, 5 x SSC, 1% (w/v) SDS at 37°C for 16 hours. The filters were then washed in 5 x SSC, 1% (w/v) SDS at 65°C for 1 hour.

The signals were visualized following the protocol of the DIG DNA labelling and detection kit.

Twelve strongly-hybridizing plaques were picked into PSB and rescreened to isolate pure plaques. The plasmids contained in the λZAPII bacteriophage vector were rescued and digested with EcoRI/XhoI to release the cDNA inserts. Most of the twelve clones contained cDNA inserts of approximately 1.8 kb. One clone, THT52, contained the longest 5' non-coding-region sequence. The complete sequence of the torenia cDNA clone (THT52), contained in the plasmid pTHT52, was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989). The sequence (SEQ ID NO:18) contained an open reading frame of 1524 bases which encodes a putative polypeptide of 508 amino acids (SEQ ID NO:19).

The nucleotide and predicted amino acid sequences of the torenia THT52 cDNA clone (SEQ ID NO:18 and SEQ ID NO:19) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2). The torenia THT52 cDNA clone showed 63.6% similarity, over 1694 nucleotides, and 67.4% similarity, over 515 amino acids, to that of the petunia OGR-38 cDNA clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 30- The F3'H assay of the torenia THT cDNA clone expressed in yeast

Construction of pYTHT6

The plasmid pYTHT6 (Figure 19) was constructed by cloning the cDNA insert from pTHT6

in a "sense" orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter of pYE22m (Tanaka *et al.*, 1988). The plasmid pTHT6 contained the THT6 cDNA clone. THT6 is identical to THT52, except that its 5' non-coding region is 75 bp shorter .

The 1.7kb THT6 cDNA insert was released from the plasmid pTHT6 upon digestion with EcoRI/XhoI. The THT6 cDNA fragment was isolated, purified and ligated with EcoRI/SalI ends of pYE22m to produce pYTHT6.

Yeast transformation, preparation of yeast extracts and the F3'H assay are described in Example 6.

F3'H activity was detected in extracts of G1315/pYTHT6, but not in extracts of non-transgenic yeast. From this it was concluded that the THT6 cDNA insert contained in pYTHT6, encoded a F3'H.

EXAMPLE 31- Isolation of a putative F3'H cDNA clone from *Pharbitis nil* (Japanese morning glory)

In order to isolate a morning glory F3'H cDNA clone, the petunia Ht1-linked F3'H cDNA clone (OGR-38), contained in pCGP1805, was used to screen a Japanese morning glory petal cDNA library, under low stringency conditions.

Construction of Japanese morning glory petal cDNA library

The petal cDNA library from young petals of *Pharbitis nil* (Japanese morning glory) was obtained from Dr Iida (National Institute of Basic Biology, Japan).

Screening of Japanese morning glory petal cDNA library

Lifts of a total of 200,000 of the amplified Japanese morning glory petal cDNA library were screened with DIG-labelled fragments of the 1.8 kb OGR-38 cDNA insert from pCGP1805. A DIG DNA labelling and detection kit from Boehringer-Mannheim was used according to

the manufacturer's recommendations.

Hybridizations were carried out in 30% (v/v) formamide, 5 x SSC, 1% (w/v) SDS at 37°C for 16 hours. The filters were then washed in 5 x SSC, 1% (w/v) SDS at 65°C for 1 hour. The signals were visualized following the protocol of the DIG DNA labelling and detection kit.

Twenty strongly-hybridizing plaques were picked into PSB and rescreened to isolate pure plaques. The plasmids contained in the λZAPII bacteriophage vector were rescued and digested with EcoRI/XhoI to release the cDNA inserts. One clone (MHT85) contained a 1.8kb insert. The complete sequence of the Japanese morning glory cDNA clone (MHT85) (SEQ ID NO:20), contained in the plasmid pMHT85, was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989). The MHT85 sequence appears to be 5 bases short of "full-length".

The nucleotide and predicted amino acid sequences of the Japanese morning glory MHT85 cDNA clone (SEQ ID NO:20 and SEQ ID NO:21) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2). The Japanese morning glory MHT85 cDNA clone showed 69.6% similarity, over 869 nucleotides, and 74.8% similarity, over 515 amino acids, to that of the petunia OGR-38 cDNA clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 32- Isolation of a putative F3'H cDNA clone from *Gentiana triflora*

In order to isolate a gentian F3'H cDNA clone, the petunia Ht1-linked F3'H cDNA clone (OGR-38), contained in pCGP1805, was used to screen a *Gentiana triflora* Pall. var *japonica* Hara petal cDNA library, under low stringency conditions.

Construction of gentian petal cDNA library

A petal cDNA library was prepared from *Gentiana triflora* Pall. var *japonica* Hara flowers, as described by Tanaka *et al.*, 1996.

Screening of gentian petal cDNA library

Lifts of a total of 200,000 of the amplified gentian petal cDNA library were screened with DIG-labelled fragments of the 1.8 kb OGR-38 cDNA insert from pCGP1805. A DIG DNA labelling and detection kit from Boehringer-Mannheim was used according to the manufacturer's recommendations.

Hybridizations were carried out in 30% (v/v) formamide, 5 x SSC, 1% (w/v) SDS at 37°C for 16 hours. The filters were then washed in 5 x SSC, 1% (w/v) SDS at 65°C for 1 hour. The signals were visualized following the protocol of the DIG DNA labelling and detection kit.

Fifteen strongly-hybridizing plaques were picked into PSB and rescreened to isolate pure plaques. The plasmids contained in the λZAPII bacteriophage vector were rescued and digested with EcoRI/XhoI to release the cDNA inserts. One clone (GHT13) contained a 1.8kb insert. The sequence of the partial gentian cDNA clone (GHT13) (SEQ ID NO:22), contained in the plasmid pGHT13, was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989).

The nucleotide and predicted amino acid sequences of the gentian GHT13 cDNA clone (SEQ ID NO:22 and SEQ ID NO:23) were compared with those of the petunia OGR-38 F3'H cDNA clone. The gentian GHT13 cDNA clone showed 68.3% similarity, over 1519

nucleotides, and 71.8% similarity, over 475 amino acids, to that of the petunia OGR-38 cDNA clone.

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

EXAMPLE 33- Isolation of putative F3'H cDNA clone from lisianthus

In order to isolate a lisianthus F3'H cDNA clone, the petunia Ht1-linked F3'H cDNA clone (OGR-38), contained in pCGP1805, was used to screen a lisianthus petal cDNA library, under low stringency conditions.

Construction and screening of lisianthus petal cDNA library

10,000 pfus of a lisianthus petal cDNA library described by Davies *et al.* (1993) and Markham and Offman (1993) were plated onto LB plates (Sambrook *et al.*, 1989) at a density of 3,000 pfus per 15 cm plate after transfecting Y1090r-, and incubated at 37°C for 16 hours. After incubation at 4°C for one hour, duplicate lifts were taken onto Hybond N+™filters (Amersham) and treated as recommended by the manufacturer.

The duplicate lifts from the lisianthus line #54 petal cDNA library were screened with ^{32}P-labelled fragments of the 1.8 kb Asp718/BamHI insert from pCGP1805.

Hybridization conditions included a prehybridization step in 1mM EDTA (pH8.0), 0.5MNa_2HPO_4 (pH7.2), 7% (w/v) SDS (Church and Gilbert, 1984) at 55°C for at least 1 hour. The ^{32}P-labelled fragments ($1x10^6$cpm/mL) were then added to the hybridization solution and hybridization was continued at 55°C for a further 16 hours. The filters were then washed in 2 x SSC, 0.1% (w/v) SDS at 55°C for 2 x 15 minutes, and exposed to Kodak

BioMax™film with an intensifying screen at -70°C for 18 hours.

Twelve strongly-hybridizing plaques were picked into PSB (Sambrook *et al.*, 1989) and rescreened to isolate purified plaques, using the hybridization conditions as described for the initial screening of the cDNA library. Sequence data were generated from the 3' and 5' ends of the cDNA inserts of four clones.

Based on sequence comparisons, pL3-6 showed similarity with the petunia OGR-38 F3'H cDNA clone and was further characterised.

The 2.2 kb cDNA insert, contained in pL3-6, was subsequently found to contain 3 truncated cDNA clones, the longest (L3-6) having high sequence similarity to the petunia OGR-38 cDNA sequence. The sequence of this L3-6 partial cDNA clone contained in the plasmid pL3-6 was determined by compilation of sequence from subclones of the L3-6 cDNA insert (SEQ ID NO:24).

The nucleotide and predicted amino acid sequences of the lisianthus L3-6 cDNA clone (SEQ ID NO:24 and SEQ ID NO:25) were compared with those of the petunia OGR-38 F3'H cDNA clone (SEQ ID NO:1 and SEQ ID NO:2). The sequence of the lisianthus L3-6 cDNA clone showed 71.4% similarity, over 1087 nucleotides, and 74.6% similarity, over 362 amino acids, to that of the petunia OGR-38 F3'H cDNA clone .

An alignment of the petunia, carnation, snapdragon, arabidopsis, rose, chrysanthemum and torenia sequences, all of which are disclosed in this specification, and various summaries of comparisons of sequence similarities among the nucleotide and corresponding amino acid sequences, can be found in Table 7 and in Tables 8, 9, 10, 11 and 12, respectively. These Tables are in Example 34, at the end of the specification.

Further investigation of the remaining clones isolated from the screening of the lisianthus

library identified another putative F3'H cDNA clone (L3-10), contained in the plasmid pL3-10. The L3-10 cDNA insert is approximately 1.8kb and appears to represent a "full-length" clone.

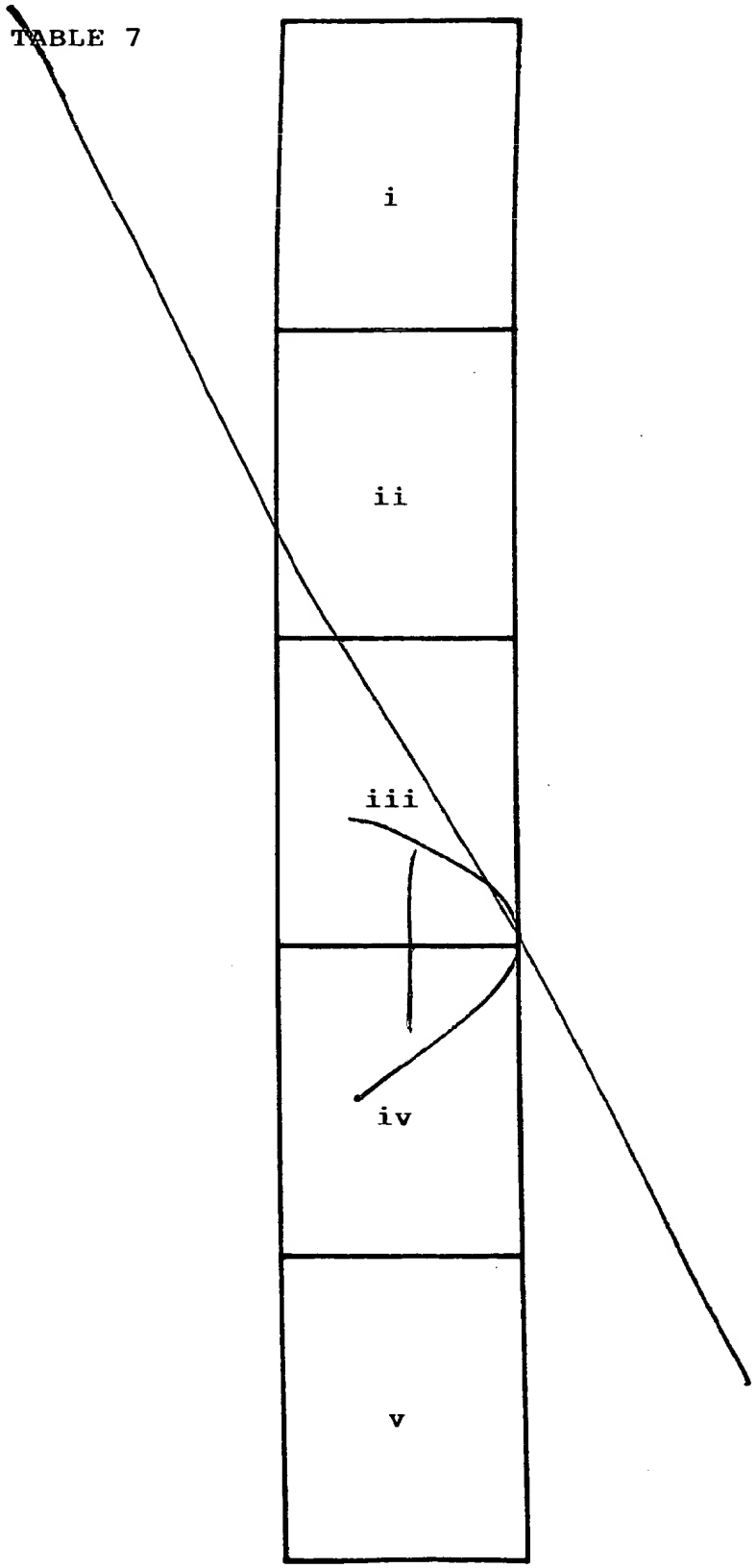
EXAMPLE 34-Alignments and comparisons among nucleotide and amino acid sequences disclosed herein

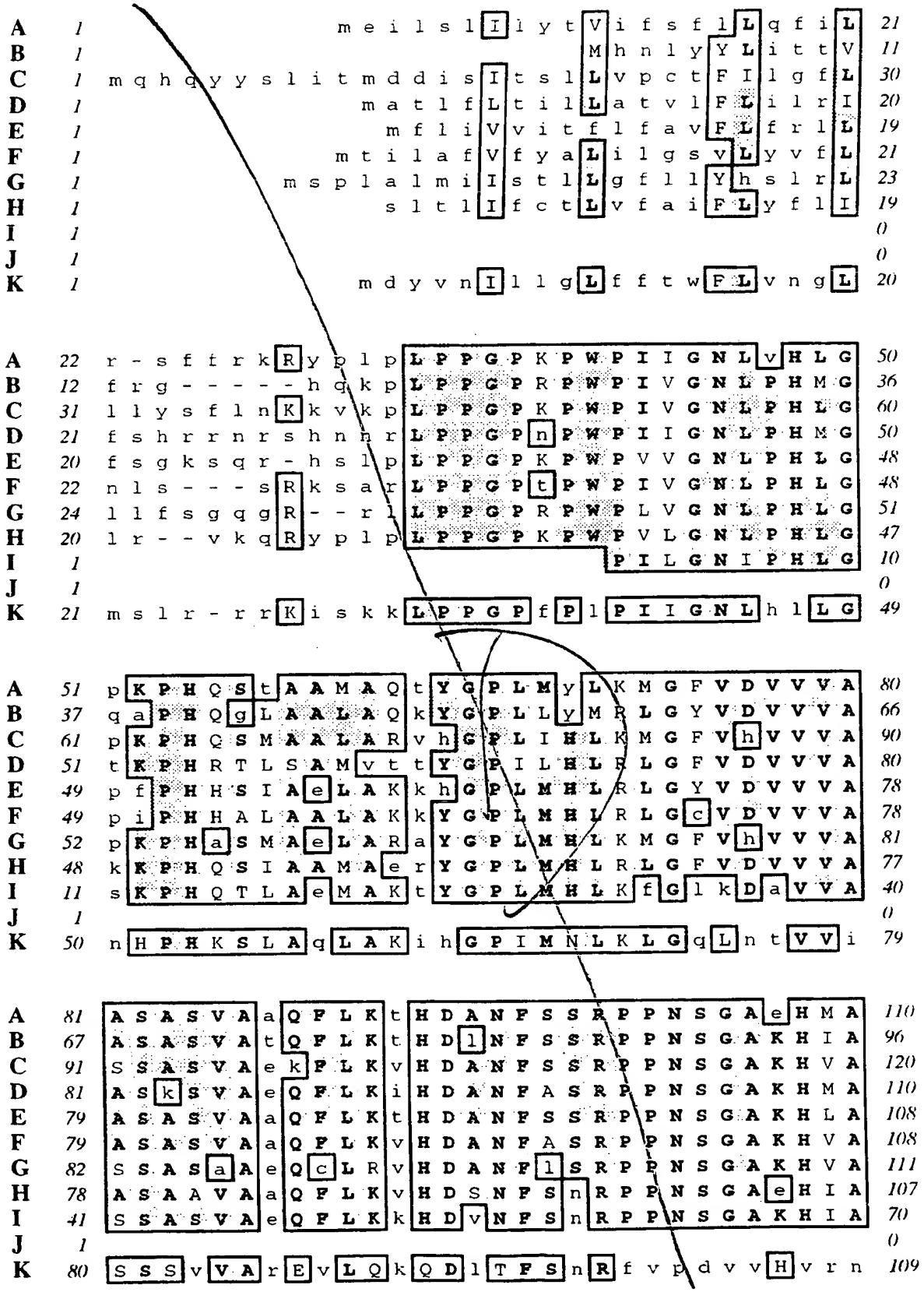
Sub B4

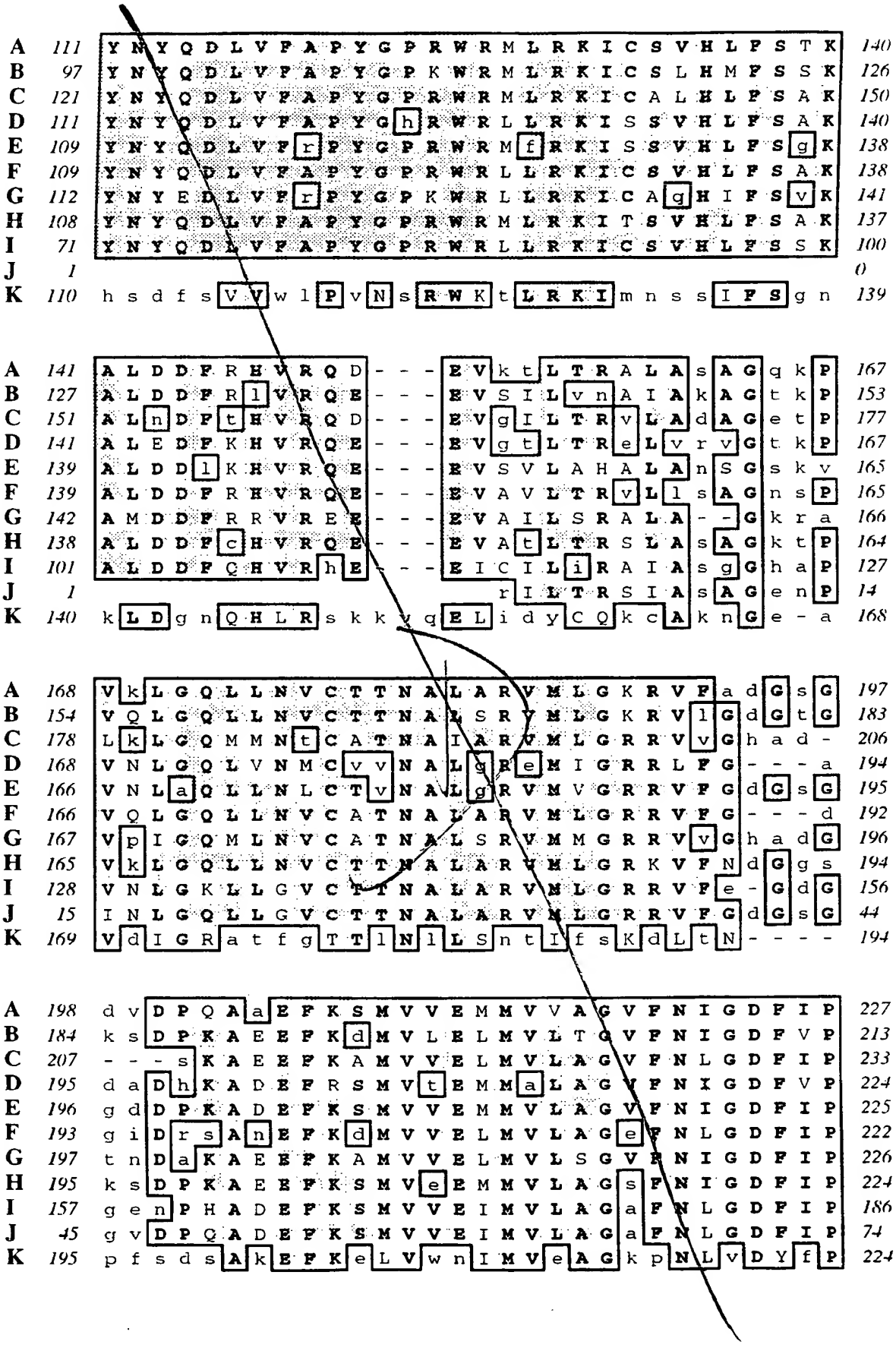
Multiple sequence alignments were performed using the ClustalW program as described in Example 3. Table 7 (below) provides a multiple sequence alignment of the predicted amino acid sequences of petunia OGR-38 (A); carnation (B); snapdragon (C); arabidopsis Tt7 coding region (D); rose (E) chrysanthemum (F); torenia (G); morning glory (H); gentian (partial sequence) (I); lisianthus (partial sequence) (J) and the petunia 651 cDNA (K). Conserved amino acids are shown in bolded capital letters and are boxed and shaded. Similar amino acids are shown in capital letters and are only lightly shaded, and dissimilar amino acids are shown in lower case letters.

Nucleotide and amino acid sequences of the F3'H cDNA clones from the above mentioned species and the coding region of the genomic clone from arabidopsis were compared using the LFASTA program, as described in Example 3. Summaries of similarity comparisons are presented in Tables 8 to 12, below.

TABLE 7



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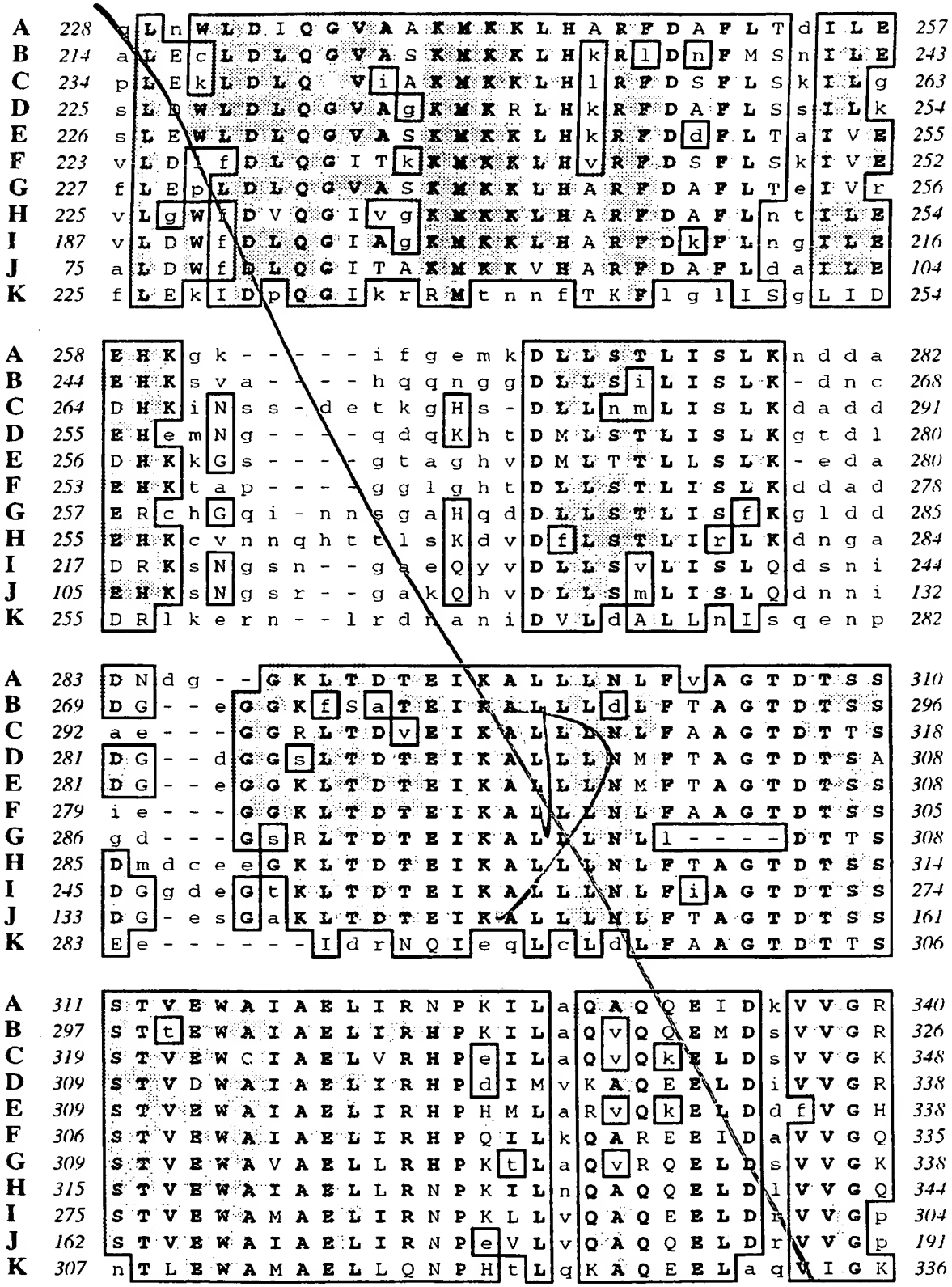
B 97 Y N Y Q D L V F A P Y G P K W R M L R K I C S L H M F S S K 126
C 121 Y N Y Q D L V F A P Y G P R W R M L R K I C A L H L F S A K 150
D 111 Y N Y Q D L V F A P Y G h R W R L L R K I S S V H L F S A K 140
E 109 Y N Y Q D L V F r P Y G P R W R M f R K I S S V H L F S g K 138
F 109 Y N Y Q D L V F A P Y G P R W R L L R K I C S V H L F S A K 138
G 112 Y N Y E D L V F r P Y G P K W R L L R K I C A g H I F S v K 141
H 108 Y N Y Q D L V F A P Y G P R W R M L R K I T S V H L F S A K 137
I 71 Y N Y Q D L V F A P Y G P R W R L L R K I C S V H L F S S K 100
J 1 0
K 110 h s d f s V V w l P v N s R W K t L R K I m n s s I F S g n 139
A 141 A L D D F R H V R Q D - - - E V k t L T R A L A s A G q k P 167
B 127 A L D D F R l V R Q E - - - E V S I L v n A I A k A G t k P 153
C 151 A L n D F t H V R Q D - - - E V g I L T R v L A d A G e t P 177
D 141 A L E D F K H V R Q E - - - E V g t L T R e L v r v G t k P 167
E 139 A L D D l K H V R Q E - - - E V S V L A H A L A n S G s k v 165
F 139 A L D D F R H V R Q E - - - E V A V L T R v L l s A G n s P 165
G 142 A M D D F R R V R E E - - - E V A I L S R A L A - - G k r a 166
H 138 A L D D F c H V R Q E - - - E V A t L T R S L A s A G k t P 164
I 101 A L D D F Q H V R h E - - - E I C I L i R A I A s g G h a P 127
J 1 r I L T R S I A s A G e n P 14
K 140 k L D g n Q H L R s k k v q E L i d y C Q k c A k n G e - a 168
A 168 V k L G Q L L N V C T T N A L A R V M L G K R V F a d G s G 197
B 154 V Q L G Q L L N V C T T N A L S R V M L G K R V l G d G t G 183
C 178 L k L G Q M M N t C A T N A I A R V M L G R R V v G h a d - 206
D 168 V N L G Q L V N M C v v N A L g R e M I G R R L F G - - - a 194
E 166 V N L a Q L L N L C T v N A L g R V M V G R R V F G d G s G 195
F 166 V Q L G Q L L N V C A T N A L A R V M L G R R V F G - - - d 192
G 167 V p I G Q M L N V C A T N A L S R V M M G R R V v G h a d G 196
H 165 V k L G Q L L N V C T T N A L A R V M L G R K V F N d G g s 194
I 128 V N L G K L L G V C T T N A L A R V M L G R R V F e - G d G 156
J 15 I N L G Q L L G V C T T N A L A R V M L G R R V F G d G s G 44
K 169 V d I G R a t f g T T l N l L S n t I f s K d L t N - - - - 194
A 198 d v D P Q A a E F K S M V V E M M V V A G V F N I G D F I P 227
B 184 k s D P K A E E F K d M V L E L M V L T G V F N I G D F V P 213
C 207 - - - s K A E E F K A M V V E L M V L A G V F N L G D F I P 233
D 195 d a D h K A D E F R S M V t E M M a L A G V F N I G D F V P 224
E 196 g d D P K A D E F K S M V V E M M V L A G V F N I G D F I P 225
F 193 g i D r s A n E F K d M V V E L M V L A G e F N L G D F I P 222
G 197 t n D a K A E E F K A M V V E L M V L S G V F N I G D F I P 226
H 195 k s D P K A E E F K S M V e E M M V L A G s F N I G D F I P 224
I 157 g e n P H A D E F K S M V V E I M V L A G a F N L G D F I P 186
J 45 g v D P Q A D E F K S M V V E I M V L A G a F N L G D F I P 74
K 195 p f s d s A k E F K e L V w n I M V e A G k p N L v D Y f P 224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D 225 sLDWLDLQGVAgKMKRLHkRFDAFLSsILk 254
E 226 sLEWLDLQGVASKMKKLHkRFDdFLTaIVE 255
F 223 vLDLfDLQGITkKMKKLHvRFDSFLSkIVE 252
G 227 fLEpLDLQGVASKMKKLHARFDAFLTeIVr 256
H 225 vLgWfDVQGIvgKMKKLHARFDAFLntILE 254
I 187 vLDWfDLQGIAgKMKKLHARFDkFLngILE 216
J 75 aLDWfDLQGITAKMKKVHARFDAFLdaILE 104
K 225 fLEkIDpQGIkrRMtnnfTKFlglISgLID 254
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C 264 DHKiNss-detkgHs-DLLnmLISLKdadd 291
D 255 EHemNg-----qdqKhtDMLSTLISLKgtdl 280
E 256 DHKkGs-----gtaghvDMLTTLLSLK-eda 280
F 253 EHKtap-----gglghtDLLSTLISLKddad 278
G 257 ERchGqi-nnsgaHqdDLLSTLISfKgldd 285
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J 105 EHKsNgsr--gakQhvDLLSmLISLQdnni 132
K 255 DRlkernn--lrdnaniDVLdALLnIsqenp 282
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B 269 DG--eGGKfSaTEIKALLLdLFTAGTDTSS 296
C 292 ae---GGRLTDvEIKALLLNLFAAGTDTTS 318
D 281 DG--dGGsLTDTEIKALLLNMFTAGTDTSA 308
E 281 DG--eGGKLTDTEIKALLLNMFTAGTDTSS 308
F 279 ie---GGKLTDTEIKALLLNLFAAGTDTSS 305
G 286 gd---GsRLTDTEIKALLLNLl----DTTS 308
H 285 DmdceeGKLTDTEIKALLLNLFTAGTDTSS 314
I 245 DGgdeGtKLTDTEIKALLLNLFiAGTDTSS 274
J 133 DG-esGaKLTDTEIKALLLNLFTAGTDTSS 161
K 283 Ee-------IdrNQIeqLcLdLFAAGTDTTS 306
A 311 STVEWAIAELIRNPKILaQAQQEIDkVVGR 340
B 297 STtEWAIAELIRHPKILaQvQQEMDsVVGR 326
C 319 STVEWCIAELVRHPeILaQvQkELDsVVGK 348
D 309 STVDWAIAELIRHPdIMvKAQEELDiVVGR 338
E 309 STVEWAIAELIRHPHMLaRvQkELDdfVGH 338
F 306 STVEWAIAELIRHPQILkQAREEIDaVVGQ 335
G 309 STVEWAVAELLRHPKtLaQvRQELDsVVGK 338
H 315 STVEWAIAELLRNPKILnQAQQELDlVVGQ 344
I 275 STVEWAMAELIRNPKLLvQAQEELDhVVGp 304
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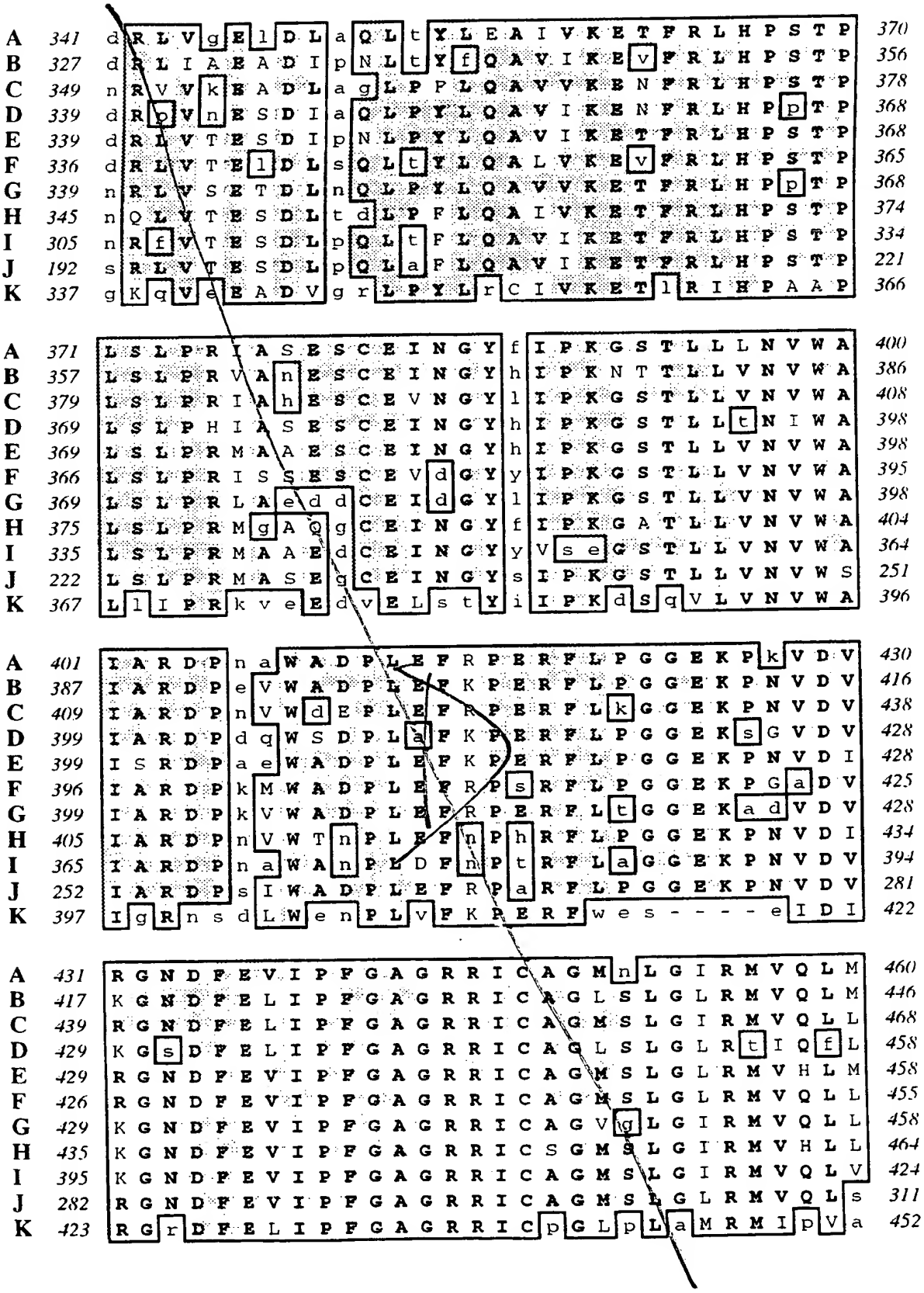
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C 349 n R V V k E A D L a g L P P L Q A V V K E N F R L H P S T P 378
D 339 d R p V n E S D I a Q L P Y L Q A V I K E N F R L H P p T P 368
E 339 d R L V T E S D I p N L P Y L Q A V I K E T F R L H P S T P 368
F 336 d R L V T E l D L s Q L t Y L Q A L V K E v F R L H P S T P 365
G 339 n R L V S E T D L n Q L P Y L Q A V V K E T F R L H P p T P 368
H 345 n Q L V T E S D L t d L P F L Q A I V K E T F R L H P S T P 374
I 305 n R f V T E S D L p Q L t F L Q A V I K E T F R L H P S T P 334
J 192 s R L V T E S D L p Q L a F L Q A V I K E T F R L H P S T P 221
K 337 g K q V e E A D V g r L P Y L r C I V K E T l R I H P A A P 366
A 371 L S L P R I A S E S C E I N G Y f I P K G S T L L L N V W A 400
B 357 L S L P R V A n E S C E I N G Y h I P K N T T L L V N V W A 386
C 379 L S L P R I A h E S C E V N G Y l I P K G S T L L V N V W A 408
D 369 L S L P H I A S E S C E I N G Y h I P K G S T L L t N I W A 398
E 369 L S L P R M A A E S C E I N G Y h I P K G S T L L V N V W A 398
F 366 L S L P R I S S E S C E V d G Y y I P K G S T L L V N V W A 395
G 369 L S L P R L A e d d C E I d G Y l I P K G S T L L V N V W A 398
H 375 L S L P R M g A Q g C E I N G Y f I P K G A T L L V N V W A 404
I 335 L S L P R M A A E d C E I N G Y y V s e G S T L L V N V W A 364
J 222 L S L P R M A S E g C E I N G Y s I P K G S T L L V N V W S 251
K 367 L l I P R k v e E d v E L s t Y i I P K d S q V L V N V W A 396
A 401 I A R D P n a W A D P L E F R P E R F L P G G E K P k V D V 430
B 387 I A R D P e V W A D P L E F K P E R F L P G G E K P N V D V 416
C 409 I A R D P n V W d E P L E F R P E R F L k G G E K P N V D V 438
D 399 I A R D P d q W S D P L a F K P E R F L P G G E K s G V D V 428
E 399 I S R D P a e W A D P L E F K P E R F L P G G E K P N V D I 428
F 396 I A R D P k M W A D P L E F R P s R F L P G G E K P G a D V 425
G 399 I A R D P k V W A D P L E F R P E R F L t G G E K a d V D V 428
H 405 I A R D P n V W T n P L E F n P h R F L P G G E K P N V D I 434
I 365 I A R D P n a W A n P L D F n P t R F L a G G E K P N V D V 394
J 252 I A R D P s I W A D P L E F R P a R F L P G G E K P N V D V 281
K 397 I g R n s d L W e n P L v F K P E R F w e s - - - - e I D I 422
A 431 R G N D F E V I P F G A G R R I C A G M n L G I R M V Q L M 460
B 417 K G N D F E L I P F G A G R R I C A G L S L G L R M V Q L M 446
C 439 R G N D F E L I P F G A G R R I C A G M S L G I R M V Q L L 468
D 429 K G s D F E L I P F G A G R R I C A G L S L G L R t I Q f L 458
E 429 R G N D F E V I P F G A G R R I C A G M S L G L R M V H L M 458
F 426 R G N D F E V I P F G A G R R I C A G M S L G L R M V Q L L 455
G 429 K G N D F E V I P F G A G R R I C A G V g L G I R M V Q L L 458
H 435 K G N D F E V I P F G A G R R I C S G M S L G I R M V H L L 464
I 395 K G N D F E V I P F G A G R R I C A G M S L G I R M V Q L V 424
J 282 R G N D F E V I P F G A G R R I C A G M S L G L R M V Q L s 311
K 423 R G r D F E L I P F G A G R R I C p G L p L a M R M I p V a 452

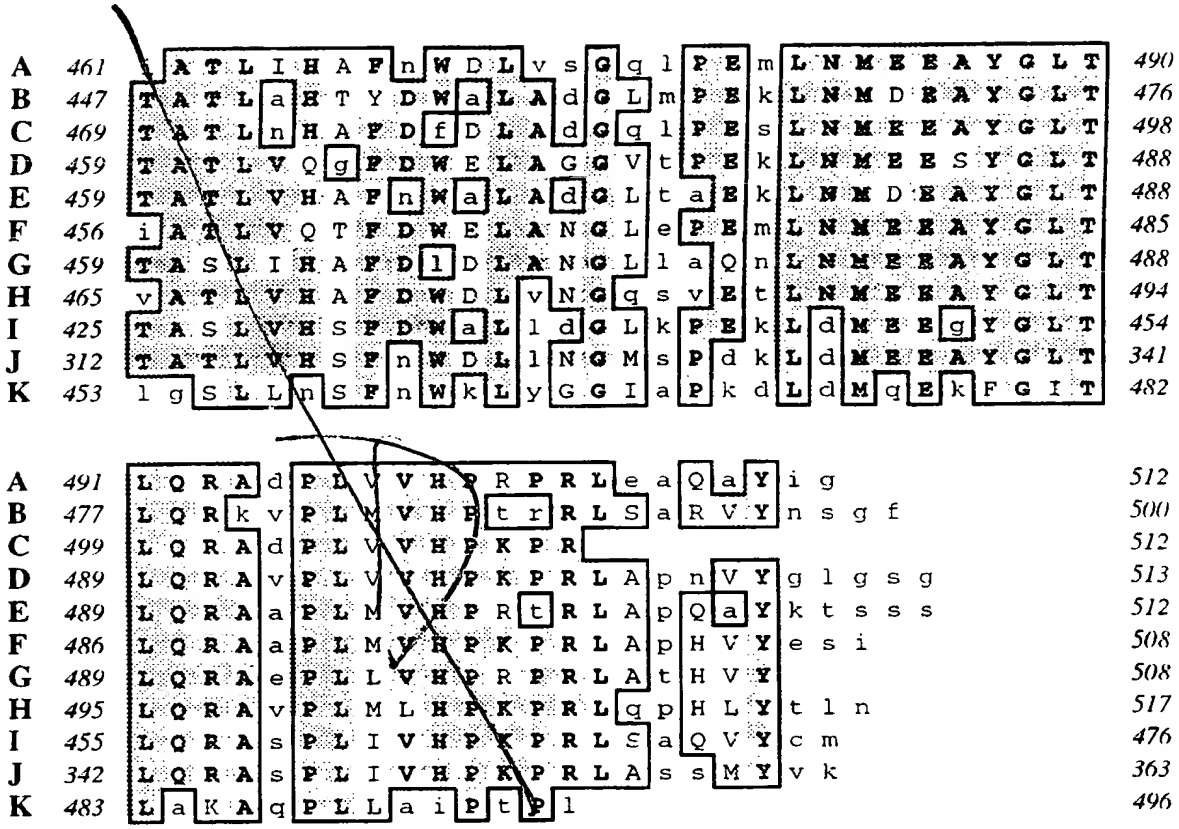
A 461 ATLIHAFnWDLvsGqlPEmLNMEEAYGLT 490
B 447 TATLaHTYDWaLAdGLmPEkLNMDEAYGLT 476
C 469 TATLnHAFDfDLAdGqlPEsLNMEEAYGLT 498
D 459 TATLVQgFDWELAGGVtPEkLNMEESYGLT 488
E 459 TATLVHAFnWaLAdGLtaEkLNMDEAYGLT 488
F 456 iATLVQTFDWELANGLePEmLNMEEAYGLT 485
G 459 TASLIHAFDlDLANGLlaQnLNMEEAYGLT 488
H 465 vATLVHAFDWDLvNGqsvEtLNMEEAYGLT 494
I 425 TASLVHSFDWaLldGLkPEkLdMEEgYGLT 454
J 312 TATLVHSFnWDLlNGMsPdkLdMEEAYGLT 341
K 453 lgSLLnSFnWkLyGGIaPkdLdMqEkFGIT 482
A 491 LQRAdPLVVHPRPRLeaQaYig 512
B 477 LQRkvPLMVHPtrRLSaRVYnsgf 500
C 499 LQRAdPLVVHPKPR 512
D 489 LQRAvPLVVHPKPRLApnVYglgsg 513
E 489 LQRAaPLMVHPRtRLApQaYktsss 512
F 486 LQRAaPLMVHPKPRLApHVYesi 508
G 489 LQRAePLLVHPRPRLAtHVY 508
H 495 LQRAvPLMLHPKPRLqpHLYtln 517
I 455 LQRAsPLIVHPKPRLSaQVYcm 476
J 342 LQRAsPLIVHPKPRLAssMYvk 363
K 483 LaKAqPLLaiPtPl 496

TABLE 7

Percentage of sequence similarity between F3'H sequence of petunia OGR-38 and F3'H sequences from other species and other P450 molecules

Species / Clone	Number of nucleotides (nt)	Number of amino acids (aa)	%similarity to OGR-38 / no. nt (area of similarity)	%similarity to OGR-38 / no. aa (area of similarity)
Petunia OGR-38	**1789nt**	**512aa**		
Snapdragon F3'H cDNA	1711nt	512aa	69.0% /1573nt (19-1578)	72.2% /507aa (1-504)
Arabidopsis partial F3'H cDNA	971nt	270aa	64.7% /745nt (854-1583)	63.7% /248aa (269-510)
Arabidopsis Tt7 coding region	1774nt	513aa	65.4% /1066nt	67.1%/511aa
Carnation F3'H cDNA	1745nt	496aa	67.3% /1555nt (28-1571)	71.5%/488aa (17-503)
Rose F3'H cDNA	1748nt	513aa	64.7% /1651nt (56-1699)	72.7%/509aa (7-510)
Gentian partial F3'H cDNA	1667nt	476aa	68.3%/1519nt (170-1673)	71.8%/475aa (40-510)
Morning Glory F3'H cDNA	1824nt	517aa	69.6%/869nt (60-1000)	74.8%/515aa (3-510)
Chrysanthemum F3'H cDNA	1660nt	508aa	68.5%/1532nt (50-1580)	73.6%/511aa (1-510)
Lisianthus partial F3'H cDNA	1214nt	363aa	71.4%/1087nt (520-1590)	74.6%/362aa (160-510)
Torenia F3'H cDNA	1815nt	508aa	63.6%/1694nt (90-1780)	67.4%/515aa (1-510)
Petunia Hf1 cDNA	1812nt	508aa	58.9% /1471nt (29-1474)	49.9% /513aa (1-511)
Petunia Hf2 cDNA	1741nt	508aa	58.9% /1481nt (37-1498)	49.1%/511aa (3-510)
Petunia 651 cDNA	1716nt	496aa	53.5% /1284nt (50-1309)	38.0% /502aa (7-503)
Mung Bean C4H cDNA	1766nt	505aa	56.0% /725nt (703-1406)	29.2% /511aa (1-503)

TABLE 8

Percentage of sequence similarity between F3'H sequence of Snapdragon and F3'H sequences from other species and other P450 molecules

Species / Clone	Number of nucleotides (nt)	Number of amino acids (aa)	%similarity to snapdragon/no. nt	%similarity to snapdragon/no. aa
Snapdragon	**1711nt**	**512aa**		
Petunia OGR-38 F3'H cDNA	1789nt	512aa	69.0% /1573nt	72.2% /507aa
Arabidopsis partial F3'H cDNA	971nt	270aa	64.5% /740nt	60.4% /240aa
Carnation F3'H cDNA	1745nt	496aa	66.7% /1455nt	68.4%/487aa
Torenia F3'H cDNA	1815nt	508aa	67.6%/1603nt	70.3%/505aa
Rose F3'H cDNA	1748nt	513aa	67.2%/1507nt	68.9%/502aa
Petunia Hf1 cDNA	1812nt	508aa	57.3% /1563nt	49.3%/491aa
Petunia Hf2 cDNA	1741nt	508aa	57.7% /1488nt	47.8%/508aa
Petunia 651 cDNA	1716nt	496aa	54.4% /1527nt	39.0% /493aa
Mung Bean C4H cDNA	1766nt	505aa	50.6% /1344nt	32.0% /490aa

TABLE 9

Percentage of sequence similarity between F3'H sequence of Arabidopsis and F3'H sequences from other species and other P450 molecules

Species / Clone	Number of nucleotides (nt)	Number of amino acids (aa)	%similarity to Arabidopsis/no. nt	%similarity to Arabidopsis/no. aa
Arabidopsis	**971nt**	**270aa**		
Petunia OGR-38 F3'H cDNA	1789nt	512aa	64.7% /745nt	63.7% /248aa
Snapdragon F3'H cDNA	1711nt	512aa	64.5%/740nt	60.4%/240aa
Carnation F3'H cDNA	1745nt	496aa	64.7% /782nt	60.6%/241aa
Rose F3'H cDNA	1748nt	513aa	68.5%/739nt	63.7%/248aa
Petunia 651 cDNA	1716nt	496aa	57.0%/521nt	40.5%/227aa
Petunia Hf1 cDNA	1812nt	508aa	58.2% /632nt	46.5% /243aa
Petunia Hf2 cDNA	1741nt	508aa	57.4% /632nt	46.1%/243aa

TABLE 10

Percentage of sequence similarity between F3'H sequence of Rose and F3'H sequences from other species and other P450 molecules

Species / Clone	Number of nucleotides (nt)	Number of amino acids (aa)	%similarity to Rose / no. nt	%similarity to Rose / no. aa
Rose	**1748bp**	**513aa**		
Petunia OGR-38 F3'H cDNA	1789bp	512aa	64.7% /1651nt	72.7%/509aa
Snapdragon F3'H cDNA	1711bp	512aa	67.2%/1507	68.9%/502aa
Carnation F3'H cDNA	1745bp	496aa	67.4%/1517nt	72.6%/486aa
Arabidopsis partial F3'H cDNA	971bp	270aa	68.5%/739nt	63.7%/248aa
Petunia 651 cDNA	1716bp	496aa	53.1%/1182nt	37.8%/502aa
Petunia Hf1 cDNA	1812bp	506aa	57%/1366nt	49.9%/503aa
Petunia Hf2 cDNA	1741bp	508aa	57.3%/1331nt	49.1%/505aa
Mung Bean C4H cDNA	1766bp	505aa	52.4%/1502nt	32.0%/510aa

TABLE 12

Percentage of sequence similarity between coding region of Arabidopsis tt7 genomic sequence and F3'H cDNA sequences from other species and other P450 molecules

Species / Clone	Number of nucleotides (nt)	Number of amino acids (aa)	%similarity to Arabidopsis tt7 / no. nt	%similarity to Arabidopsis tt7 / no. aa
Arabidopsis Tt7 coding region	**1774nt**	**513aa**		
Petunia OGR-38 F3'H cDNA	1789nt	512aa	65.4% /1066nt	67.1%/511aa
Snapdragon F3'H cDNA	1711nt	512aa	62.7%/990nt	64.9%/504aa
Carnation F3'H cDNA	1745nt	496aa	63.2%/1050nt	65.9%/495aa
Rose F3'H cDNA	1748nt	513aa	65.5%/1076nt	68%/512aa
Petunia 651 cDNA	1716nt	496aa	56.5%/990nt	36.5%/502aa
Petunia Hf1 F3'H cDNA	1812nt	506aa	56.8%/995nt	47.5%/509aa
Petunia Hf2 F3'H cDNA	1741nt	508aa	55.2%/1063nt	46.8%/509aa

Those skilled in the art, will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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